PRO-INFLAMMATORY CYTOKINES CONTROL THE ANTI-VIRAL NK CELL RESPONSE

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PRO-INFLAMMATORY CYTOKINES CONTROL THE ANTI-VIRAL NK CELL RESPONSE.

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Although natural killer (NK) cells are considered part of the innate immune system, recent studies have demonstrated the capability of virus-specific NK cells to become long-lived and contribute to potent recall responses similar to T and B cells. The precise signals that promote the generation of a long-lived NK cell response are largely undefined. This dissertation investigates the role of pro-inflammatory cytokines interleukin (IL)-12, IL-18, and type I IFN on the NK cell response during mouse cytomegalovirus (MCMV) infection. We demonstrate that IL-12 and its signaling component STAT4 are indispensible for MCMV-specific NK cell expansion and generation of "memory" NK cells in lymphoid and non-lymphoid tissues. Furthermore, IL-12 and STAT4 signaling in activated NK cells increased the expression of the adaptor protein MyD88, which mediates signaling downstream of the IL-18 receptor, and T-box transcription factor T-bet. During MCMV infection, NK cells required IL-18 receptor and MyD88 for optimal primary expansion, but not recall responses. In addition, NK cell-specific deletion of T-bet or Eomes crippled the anti-viral NK cell response. Lastly, we show type I IFN and STAT1 signaling protects NK cells form NKG2D-mediated killing, thus promoting a robust antiviral NK cell

response. This work highlights the complex, non-redundant, and stage-specific role of pro-inflammatory cytokines and transcription factors on the NK cell response.

BIOGRAPHICAL SKETCH

Sharline completed a Bachelors of Arts in biochemistry at Wellesley College and matriculated into the Weill Cornell/Rockefeller/Sloan Kettering MD-PhD Program in 2008. She joined Joseph Sun's laboratory in 2011.

For Douglas F. Dyckes, PhD.

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LIST OF ABBREVIATIONS

ADCC	Antibody dependent cellular cytotoxicity
CFSE	Carboxyfluorescein succinimidyl ester
CTV	Cell Trace Violet
GVHD	Graft-versus-host disease
CMV	Cytomegalovirus
HSCT	Hematopoietic stem cell transplant
IFN	Interferon
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation primary response 88
NK	Natural killer cell
STAT	Signal transducer and activator of transcription
Th	T helper
TRAIL	TNF-related apoptosis inducing ligand
WT	Wildtype

LIST OF SYMBOLS

α	alpha
β	beta
γ	gamma
μ	micro
°	degree

CHAPTER 1

INTRODUCTION

NK cells: Bridging the gap between innate and adaptive immunity

The immune system is traditionally compartmentalized into two distinct arms of immunity, the innate arm and the adaptive arm, which together aid in the efficient execution of anti-pathogen and anti-tumor responses. The innate immune system constitutes the earliest defense against pathogen invasion and is typically described as mediating swift, but non-specific and short-lived immune responses. Conversely, the adaptive immune system provides a delayed but antigen-specific response, and is capable of immunological memory, a quality that has become a defining feature of adaptive immunity. Historically, T and B cells, which possess antigen-specific receptors that result from somatic gene rearrangement, have been the sole members of the adaptive immune system, classifying all other immune cells as part of the innate immune system. Despite being a vital member of the innate immune system, recent findings have revealed that NK cells fail to adhere to the traditional innate-adaptive dichotomy and exhibit many "adaptive" features, including immunological memory and a heightened effector response upon antigen re-encounter.

NK cell characteristics and function

NK cells are traditionally classified as innate immune cells. Initially postulated to reject cells that have downregulated MHC I due to viral infection or transformation (termed the "missing self" hypothesis (1)), NK cells are now appreciated to form a

vital component of the anti-viral and anti-tumor immune response. NK cells are derived from the same lymphocyte progenitor as T and B cells (2), and resemble $CD8^+$ T cells in phenotype and function. Despite these commonalities, NK cells differ from T and B cells in that their function does not rely on RAG-mediated rearrangement of antigen receptors. Instead, NK cells express a combination of germ-line encoded receptors that provide inhibitory and activating signals, which ultimately dictate the activation status of the cell. Once activated, NK cells are able to lyse target cells through the exocytosis of perforin- and granzyme-containing granules, and to secrete cytokines, such as IFN-γ, TNF-α, and GM-CSF, which alert and influence the broader immune response. Until recently, scientists have considered these NK cell responses to be short-lived, antigen-non-specific, and qualitatively similar during primary or subsequent challenges with a specific pathogen. However, recent studies have ascribed a broader range of new immunological capabilities to NK cells, including the ability to execute a long-lived antigen specific response, or "memory" response – a feature previously thought only to exist in B and T cells. NK cell "memory" responses have been demonstrated in the context of challenge with haptens, pro-inflammatory cytokines, and certain viruses, and the cellular and molecular events that control these responses are an area of active investigation in the field (3).

Cytomegalovirus (CMV) infection results in an anti-viral NK cell response in both human and mice. A member of the beta subfamily of *Herpesviridae*, CMV is a large double-stranded DNA virus that is thought to have co-evolved with its mammalian host species. This virus-host co-evolution is exemplified by the

numerous viral homologues of MHC I proteins encoded by mouse CMV (MCMV) and human CMV (HCMV), which allow the virus to evade host immune cells (4). The importance of NK cells in the control of CMV is highlighted by various reports of increased susceptibility to CMV infection in mice and humans lacking functional NK cells (5-7). Furthermore, studying the NK cell response against MCMV has proven to be a useful model for further understanding HCMV infection in humans, due to the high similarity in pathogenicity of the two viruses and the similarities in clinical manifestation of disease (8).

MCMV was the first pathogen described to elicit an antigen-specific NK cell response. Following MCMV infection of C57BL/6 mice, the viral antigen m157 is expressed on the surface of infected cells, and causes the robust expansion of NK cells bearing the activating Ly49H receptor (9, 10), which signals through the DAP12 adaptor protein (11) (Figure 1.1). $Ly49H^+$ NK cells undergo a clonal-like expansion phase that peaks at day 7 post-infection (PI) (9). This "clonal"-like expansion phase is followed by a Bim-mediated contraction phase that results in a population of long-lived memory NK cells (12). Memory $Ly49H^+$ NK cells have been shown to mediate a more protective anti-viral response upon secondary challenge with MCMV (13). Long-lived NK cell responses have also been reported in humans during viral infection. Human studies have identified an enrichment of NK cells bearing the germline-encoded NKG2C receptor in patients with acute HCMV infection (14-16). Furthermore, HCMV reactivation in patients undergoing

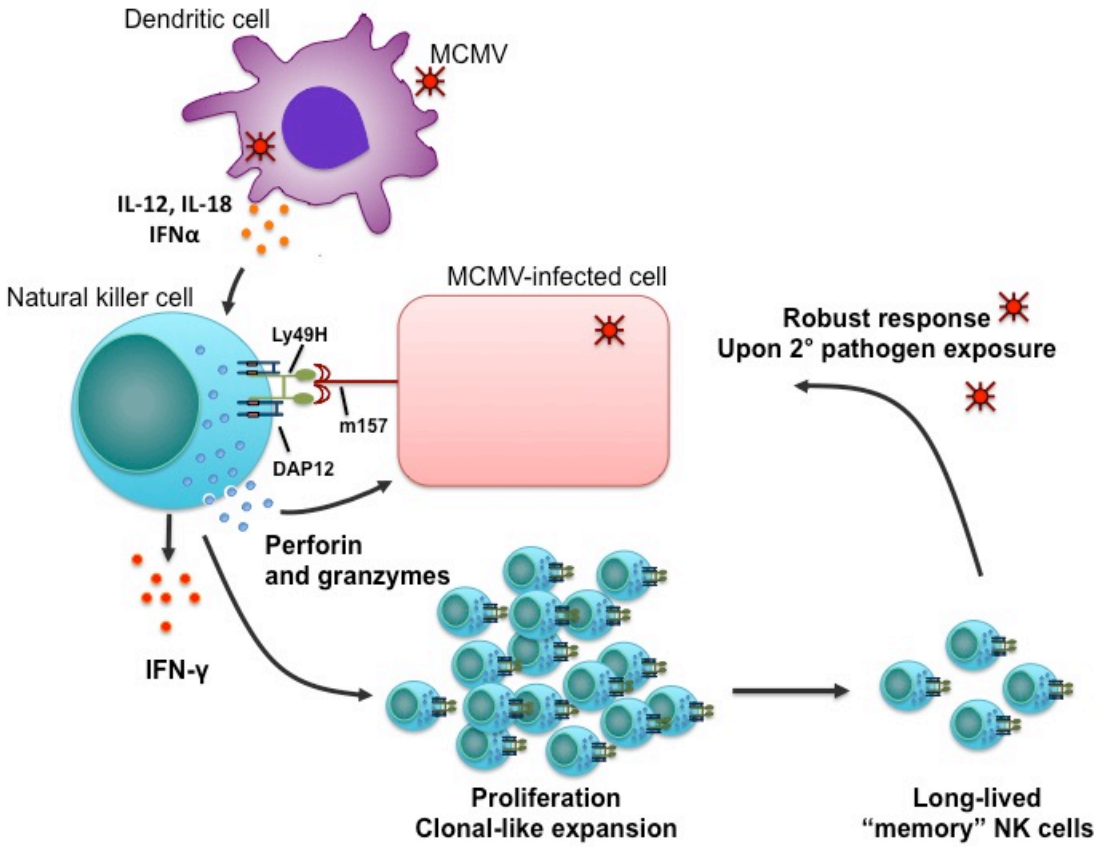


Figure 1.1. The NK cell response against MCMV. A subset of natural killer cells in the C57BL/6 mouse express the activating receptor Ly49H, which recognizes MCMV-encoded glycoprotein m157. Antigen receptor engagement, along with MCMV-induced pro-inflammatory cytokines, leads to the potent activation of the NK cell, which promotes cytokine production (IFN-γ), cell-mediated toxicity (perforin and granzymes), and the clonal-like proliferation of Ly49H-bearing NK cells. Virally expanded antigen specific NK cells undergo a contraction phase that leaves behind a pool of long-lived "memory" NK cells, which have heightened protective capabilities upon secondary pathogen encounter.

hematopoietic stem cell transplantation resulted in a long-lived population of NKG2C$^+$ NK cells that were potent IFN-γ producers (17).

The capability of an NK cell to control viral infections and to kill a susceptible target cell without prior sensitization has made this cell an attractive target in vaccine strategies and anti-tumor immunotherapy. NK cells help mediate the protective effect of certain DC-based vaccines (18). NK cell adoptive immunotherapy has also found success. NK cells have been shown to decrease graft-versus-host-disease (GVHD) and facilitate engraftment in patients receiving hematopoietic stem cell transplant (HSCT) (19). Moreover, post-transplant infusion of NK cells in AML patients reduced leukemia progression and increased overall survival (20). Although the potential benefits of NK cells in vaccination and immunotherapy have become evident, further knowledge of how to activate and harness NK cell function will prove a key step in the successful use of NK cells in the clinic.

Pro- inflammatory cytokines: A critical influence on NK cell function

NK cell development, homeostasis, and function are directly influenced by cytokines. During infection, the pro-inflammatory cytokines, IL-12, IL-18, and type I IFNs are of interest in NK cells for several reasons. First, resting NK cells constitutively express the appropriate receptors to sense these cytokines. Second, pro-inflammatory cytokines may provide a crucial signal in antigen-dependent NK cell activation. CD8$^+$ T cells require 3 signals for a productive and protective antigen-specific response (Figure 1.2). “Signal 1” is provided through the triggering

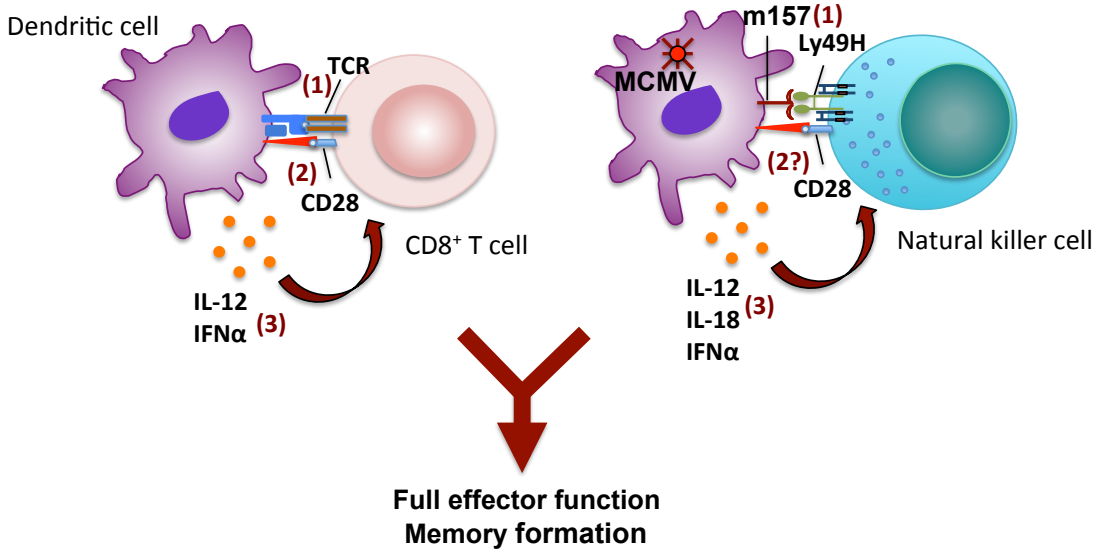


Figure 1.2. Necessary signals for optimal anti-viral responses in NK and CD8$^+$ T cells. Viral infection activates NK and CD8$^+$ T cells, which mount a specific response following the triggering of antigen receptors. The proper activation, expansion, and differentiation of CD8$^+$ T cells depends on three important signals: **(1)** antigen-mediated TCR engagement, **(2)** costimulation, and **(3)** cytokine signaling, like that provided by IL-12 or type I interferon. NK cells also rely on antigen stimulation and pro-inflammatory cytokine signaling for the development of full effector function and memory formation. Unpublished observations by Sun et al. suggest costimulation through CD28 is also necessary for optimal NK cell activation.

TCR by MHC class I plus peptide (21). Virus-specific NK cells receive a similar signal through the interaction of Ly49H with viral m157. “Signal 2” in T cells comes in the form of co-stimulation through the engagement of CD28 (21). Unpublished observations from our lab suggest CD28 is also important during NK cell activation. Lastly, a productive and long-lasting antigen-specific $CD8^+$ T cell response requires “Signal 3”, provided by pro-inflammatory cytokines IL-12 and type I IFN (22). “Signal 3” is also able to affect the gene expression landscape by regulating the expression of key transcription factors involved in cellular differentiation. More specifically, IL-12 modulates the expression of T-box transcription factors, T-bet and Eomes, which influence effector and memory $CD8^+$ T cell differentiation following pathogen infection (23, 24). IL-12, IL-18, and type I IFN are known to strongly impact the early anti-viral NK cell response (discussed below). Moreover, T-box transcription factors, T-bet and Eomes, are known to affect NK cell development and maturation; however, their effect on the mature NK cell anti-viral response is unclear. The potential effects on the long-lived antigen-specific NK cell response by “Signal 3” cytokines and the transcription factors they help modulate, have not been previously addressed.

Ex vivo cytokine treatment was recently shown to activate naïve NK cells, endowing them with enhanced effector functions and a prolonged lifespan, reminiscent of memory (25). Resting mouse NK cells treated with an IL-12, IL-18, and IL-15 cytokine cocktail persisted for weeks following-transfer into lymphopenic hosts. Moreover, these cytokine-treated NK cells produced more IFN-γ following re-stimulation than their untreated counterparts (25) – much like memory NK cells

generated during infection - and were shown to be more protective than untreated NK cells against established mouse tumors (26). Similarly, human NK cells stimulated with exogenous pro-inflammatory cytokines also exhibit enhanced IFN-γ production upon re-stimulation (27). The mechanism of cytokine-induced NK cell memory is still unknown, but may involve heritable epigenetic repatterning induced by pro-inflammatory cytokine treatment.

Several studies have explored the role of pro-inflammatory cytokines on the anti-viral NK cell response (28-31). Direct infection of cytokine- or cytokine receptor-deficient mice revealed a non-redundant role for IL-12 and IL-18 on NK cell production of IFN-γ following MCMV infection (30, 31). Other studies have suggested IL-18, but not IL-12, influences NK cell proliferation after viral infection (32). However, in light of the global effects of IL-12, IL-18, and type I IFN on the broader immune system, a more refined experimental system must be employed to address the direct effect of cytokine signaling on NK cells during viral infection. In addition, the specific role of pro-inflammatory cytokine signaling on the generation of memory NK cells in the context of viral infection has yet to be explored.

IL-12

IL-12 is a potent immunomodulatory cytokine that promotes cell-mediated immunity. IL-12 signals through a heterodimeric receptor composed of two chains: IL-12Rβ1 and IL-12Rβ2 (Figure 1.3). Engagement of the IL-12Rβ1/IL-12Rβ2 receptor by IL-12 leads to the activation of Janus family kinases, Tyrosine kinase 2 (Tyk2) and Janus kinase 2 (Jak2) (33), which in turn activate the transcription

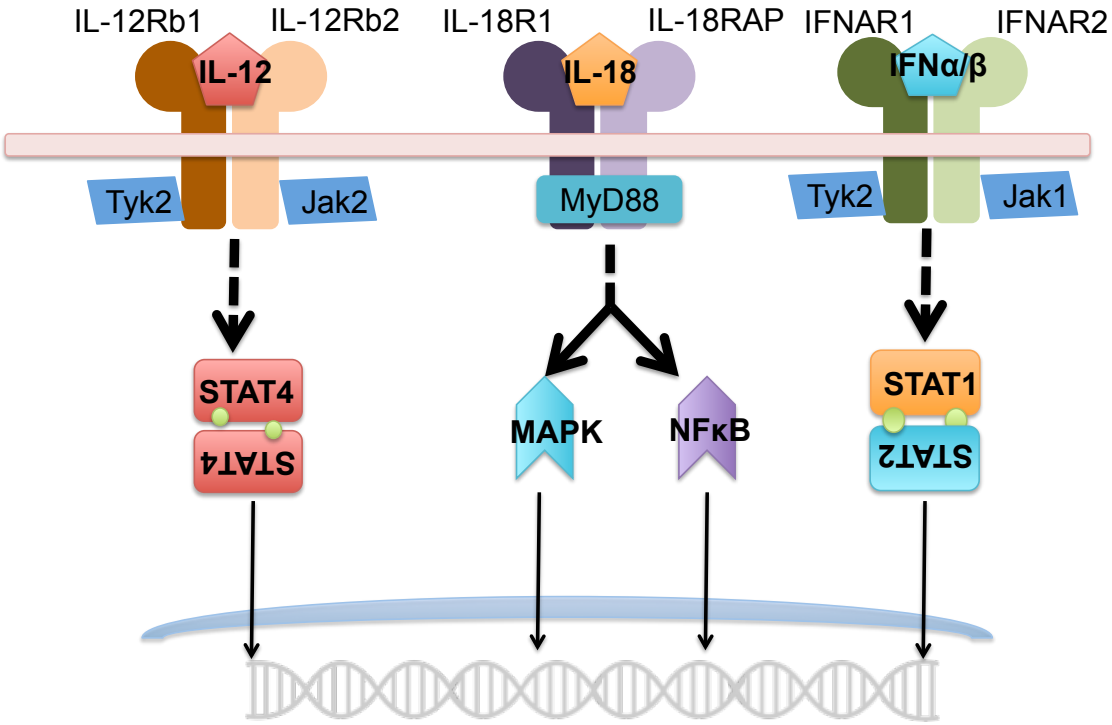


Figure 1.3. IL-12, IL-18, and type I IFN signaling pathways. Triggering of the IL-12 receptor by IL-12 leads to the recruitment and activation of Janus family kinases, Tyrosine kinase 2 (Tyk2) and Janus kinase 2 (Jak2). Once activated, Tyk2 and Jak2 primarily activate STAT4. Activation of STAT4 leads to the formation of a homodimer that translocates into the nucleus and affects gene transcription. Upon binding of IL-18, the IL-18 receptor recruits MyD88. MyD88 triggers a signaling pathway that leads to the activation of NFκB and MAPK. NFκB and MAPK are able to translocate into the nucleus and affect gene transcription. Triggering through the type I IFN receptor leads to the phosphorylation and activation of STAT1 and STAT2. Activation of the STAT proteins leads to the formation of STAT1 homodimers and STAT1/2 heterodimers that translocate to the nucleus and affect gene transcription.

factor, signal transducer and activation of transcription 4 (STAT4) (34), among others. Activation-induced phosphorylation of STAT4 causes its homodimerization and translocation into the nucleus, where it acts to regulate gene transcription (35).

IL-12 is produced early after viral infection (29-31) by antigen-presenting cells (APCs) like macrophages (36) and dendritic cells (37), and has wide reaching effects on hematopoietic cells, in particular lymphocytes. For example, IL-12 is known to promote the differentiation of naive $CD4^+$ T cells into T-helper (Th) 1 cells (38), the activation of NK and $CD8^+$ T cells, and B cell proliferation and differentiation (39). Furthermore, IL-12 shapes pathogen-induced $CD8^+$ T cell differentiation through the inverse regulation of T-box transcription factors, T-bet and Eomes (23). In NK cells, IL-12 is best known for its ability to strongly induce IFN-γ production following viral infection (40), a task it completes with help of IL-18 (41).

IL-18

IL-18 is a member of the IL-1 family of cytokines. It is produced as a pro-cytokine that is cleaved by inflammasome-activated caspase-1 into its biologically active form (42). IL-18 signals through a heterodimeric receptor composed of two chains: IL-18R1 and IL-18RAP (Figure 1.3). Unlike most other hematopoietic cells, NK cells express very high levels of both receptor chains at rest. Upon binding of IL-18, the heterodimeric receptor recruits myeloid differentiation primary response 88 (MyD88) protein, which in turn directs the activation of the NFκB and MAPK signaling pathways. Numerous cells, including dendritic cells (43), macrophages

(44), neutrophils (45), and non-hematopoietic cells (46, 47) produce IL-18. IL-18 is thought to provide resting NK cells with a "priming" signal that renders them responsive to IL-12 stimulation (48), and has been suggested to influence NK cell migratory function (49). In collaboration with IL-12, IL-18 stimulates NK, $CD8^+$ T, Th1 and B cells to secrete IFN-γ. However, IL-18 alone or in conjunction with IL-4 and IL-13 can stimulate Th2 cytokines and contribute to allergic inflammation (50-52).

Type I IFN

The type I IFN cytokine family is composed of several IFN-alpha subtypes and one IFN-beta subtype in humans and mice, among others (53). Type I IFN family cytokines signal through a ubiquitously expressed heterodimeric receptor comprised of two chains: IFNAR1 and IFNAR2 (Figure 1.3). Triggering through the type I IFN receptor leads to the phosphorylation and activation of STAT1 and STAT2, and the subsequent formation of STAT1 homodimers and STAT1/2 heterodimers that translocate to the nucleus and regulate gene transcription. Immune and non-immune cells alike produce and sense type I IFNs. Type I IFNs are able to act directly on NK cells to promote their activation and cytotoxic function during viral infection (40, 54-56). Type I IFNs were initially suggested to induce the proliferation of NK cells during MCMV infection (29). This effect, however, was not a consequence of direct signaling on NK cells, but rather a result of type I IFN induction of IL-15 (57).

T-box Transcription Factors

T-box family transcription factors Eomes and T-bet have wide-ranging effects that direct lymphocyte immunity. The role of T-bet and Eomes in the response of $CD8^+$ T cells against pathogens has been well characterized (58, 59). Recent studies have highlighted the importance of Eomes and T-bet in NK cell development and function (58, 60-62). Similar to effector and memory $CD8^+$ T cells, mature NK cells exhibit constitutive T-bet and Eomes expression (60, 63). T-bet and Eomes have been described to control specific checkpoints of NK cell development and maturation, with expression of DX5, loss of TRAIL, and gain of Ly49 receptor diversity (all characteristics of NK cell maturation) being dependent on Eomes (61). In the liver, T-bet was shown to control the development of a distinct $Eomes^-$ NK cell lineage (62). The role of these transcription factors on the antiviral response of mature NK cells has not been studied.

The work presented in this thesis seeks to explore the cell-intrinsic role of the pro-inflammatory cytokines, IL-12, IL-18, and Type I IFNs, and T-box transcription factors, T-bet and Eomes, on the early and long-lived NK cell response against MCMV. In contrast to previous reports, the studies described herein utilize experimental systems that eliminate or decrease pleiotropic effects of cytokine signaling, by employing NK cell-specific deletion of the IL-12, IL-18, or type I IFN cytokine receptors; additionally, this works employs an inducible gene deletion system to elucidate the role of T-bet and Eomes in mature NK cells during the course of MCMV infection.

CHAPTER 2

PRO-INFLAMMATORY CYTOINE SIGNALING REQUIRED FOR THE GENERATION OF NATURAL KILLER CELL MEMORY

^ *contributed equally to this paper*

Introduction

The generation of a productive NK cell response is crucial to protect the host from viral infection. In the absence of NK cells or NK cell function, both mice and humans are susceptible to a number of pathogens, particularly members of the herpesvirus family (64). Accumulating evidence in mice and humans suggests that like the cells of adaptive immunity, NK cells can "remember" previously encountered pathogens through the generation of long-lived memory cells following initial antigen exposure (65-67). During MCMV infection, Ly49H-bearing NK cells undergo a robust clonal-like expansion (13, 68) and persist in both lymphoid and non-lymphoid organs for several months (13). During a second or third encounter with the same virus, these long-lived memory NK cells are capable of prolific recall responses, mediating greater effector function and protection than naïve resting NK cells (25, 69). Similar robust NK cell clonal-like expansion and memory has been observed during acute hantavirus and HCMV infection in humans, where recent longitudinal studies revealed virus-specific responses in the NKG2C-bearing NK

cell subset (14, 70). The goal of immunization is to provide protection against subsequent infection, and thus it is vital to define the precise signals that promote the generation of NK cell memory.

Pro-inflammatory cytokines such as IL-12 are known to globally promote NK and T cell activation and cytotoxicity. Binding of IL-12 to a two chain receptor composed of IL-12 receptor (IL-12R) β1 and β2 results in a signaling cascade leading to phosphorylation and dimerization of STAT4, which translocates to the nucleus and activates downstream targets and transcription of effector cytokine genes such as IFN-γ (71). Early studies involving cytokine or neutralizing antibody treatment of mice demonstrated that IL-12 has global effects on the immune system, as many hematopoietically derived cells express the IL-12R (71). During infection, IL-12 is primarily produced by dendritic cells, and can act on many cell types including B cells, T cells, NK cells, NK T cells, and even other dendritic cells and hematopoietic progenitor cells (71). Studies comparing the response of NK cells that can or cannot sense IL-12, in a setting where pleiotrophic effects are reduced or eliminated, have not been done. Furthermore, long-lived memory NK cell generation in the absence of IL-12 signaling was not previously investigated. Using mice deficient in the IL-12 receptor and STAT4, and a recently developed adoptive transfer system (13), we were uniquely able to investigate the direct *in vivo* role of IL-12 signaling in NK cells during MCMV infection, in the absence of any indirect effects.

Results and Discussion

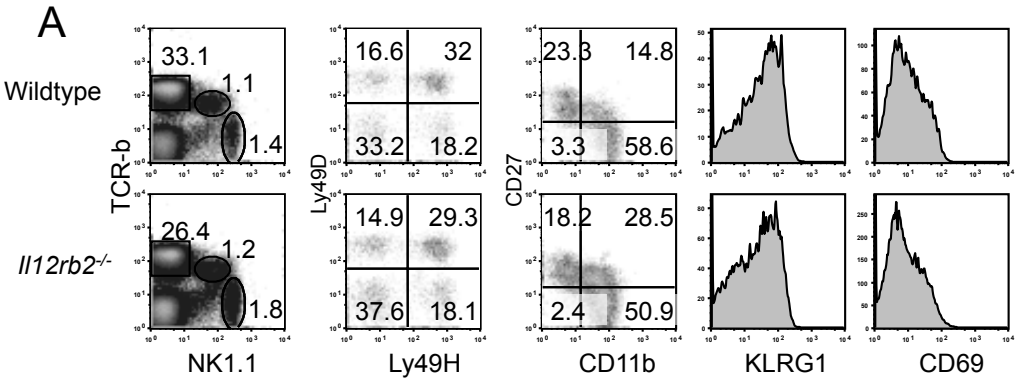
Similar phenotype and function of wildtype and Il12rb2$^{-/-}$ NK cells at steady-state

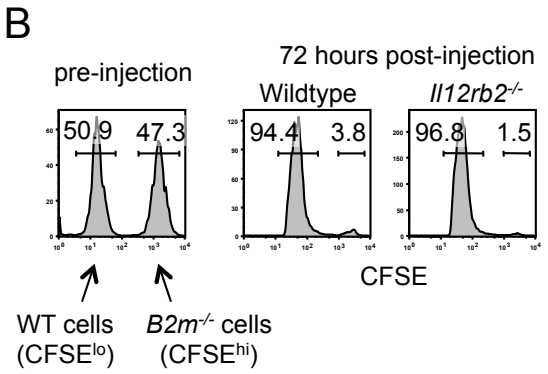
IL-12 is not required for NK cell development or homeostasis during steady-state (i.e. the absence of inflammation, infection, or lymphopenia), as normal NK cell numbers are found in IL-12- and IL-12R-deficient mice (72-75). In accordance with prior studies, *Il12rb2*$^{-/-}$ mice contained similar percentages of T, B, NK, and NK T cell populations when compared with wildtype mice (Figure 2.1A, and data not shown). Within the NK cell compartment, WT and *Il12rb2*$^{-/-}$ mice had similar percentages of Ly49D- and Ly49H-expressing cell subsets (Figure 2.1A). Furthermore, *Il12rb2*$^{-/-}$ NK cells exhibited a phenotype similar to WT NK cells as determined by CD27, CD11b, KLRG1, and CD69 expression (Figure 2.1A). In the absence of infection, WT and *Il12rb2*$^{-/-}$ mice cleared β2m-deficient target cells equally well (Figure 2.1B), suggesting that *Il12rb2*$^{-/-}$ NK cells exhibited normal *in vivo* cytotoxic function. When activating NK cell receptors were triggered with plate-bound antibodies, *Il12rb2*$^{-/-}$ NK cells were able to degranulate similar to WT NK cells (Figure 2.1C). Lastly, *Il12rb2*$^{-/-}$ NK cells were able to kill m157-bearing target cells as well as WT NK cells *ex vivo* (Figure 2.1D), demonstrating that Ly49H-mediated cytotoxicity is not dependent upon IL-12 signaling at steady state.

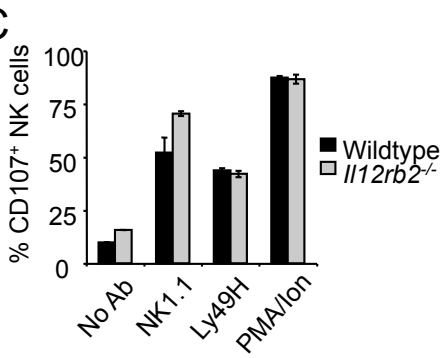
Defective expansion of Il12rb2$^{-/-}$ NK cells during MCMV infection

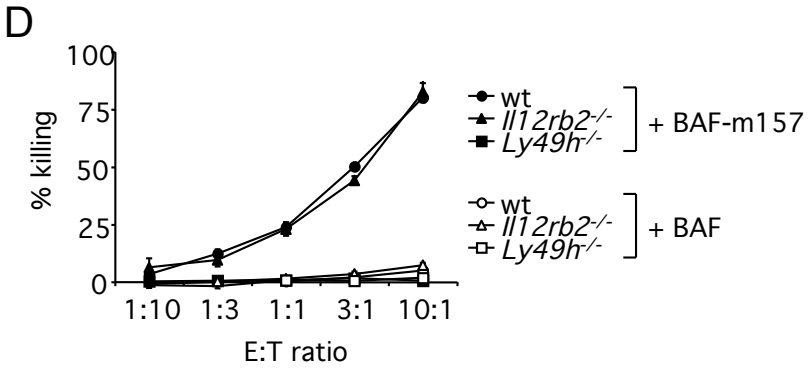
Previous studies examined the role of IL-12 on NK cells by one of three methods: injecting the IL-12 cytokine directly, blocking IL-12 with neutralizing antibodies, or infecting cytokine-deficient mice (29, 72-76). In each of these experimental

Figure 2.1. NK cells from IL-12 receptor-deficient mice are phenotypically and functionally similar to NK cells from WT mice. (**A**) Percentages of TCR-β^- NK1.1$^+$ NK, TCR-β^+ NK1.1$^+$ NKT, and TCR-β^+ NK1.1$^-$ T cell populations are shown for uninfected WT and *Il12rb2*$^{-/-}$ mice (left plot). The right plots are gated on TCR-β^- NK1.1$^+$ cells and analyzed for expression of Ly49D, Ly49H, CD27, CD11b, KLRG1, and CD69. (**B**) Wildtype and *B2m*$^{-/-}$ splenocytes were labeled with low or high concentrations of CFSE, respectively, and co-injected into WT or *Il12rb2*$^{-/-}$ mice. Transferred cells were analyzed in spleen of recipient mice 72 hours after injection. (**C**) NK cells from WT or *Il12rb2*$^{-/-}$ mice were stimulated with plate-bound antibodies against NK1.1 and Ly49H, or with PMA and ionomycin. Uncoated wells (containing no Abs) served as negative control and background staining. Percentages of TCR-β^- NK1.1$^+$ cells expressing CD107 (LAMP-1) are shown for each condition. Error bars show s.e.m. (n = 2–3 for each condition). (**D**) Varying numbers of NK cells from WT, *Il12rb2*$^{-/-}$, or *Ly49h*$^{-/-}$ mice were incubated with Ba/F3 and Ba/F3-m157 target cells labeled with ^{51}Cr. Percentage killing was calculated based on release of ^{51}Cr into supernatant by lysed target cells. All data are representative of at least 3 independent experiments.


A
Wildtype
Il12rb2-/-
TCR-b
NK1.1
33.1
1.1
1.4
26.4
1.2
1.8
Ly49D
Ly49H
16.6
32
33.2
18.2
14.9
29.3
37.6
18.1
CD27
CD11b
23.3
14.8
3.3
58.6
18.2
28.5
2.4
50.9
KLRG1
CD69


B
pre-injection
72 hours post-injection
Wildtype
Il12rb2-/-
50.9
47.3
94.4
3.8
96.8
1.5
CFSE
WT cells
(CFSElo)
B2m-/- cells
(CFSEhi)

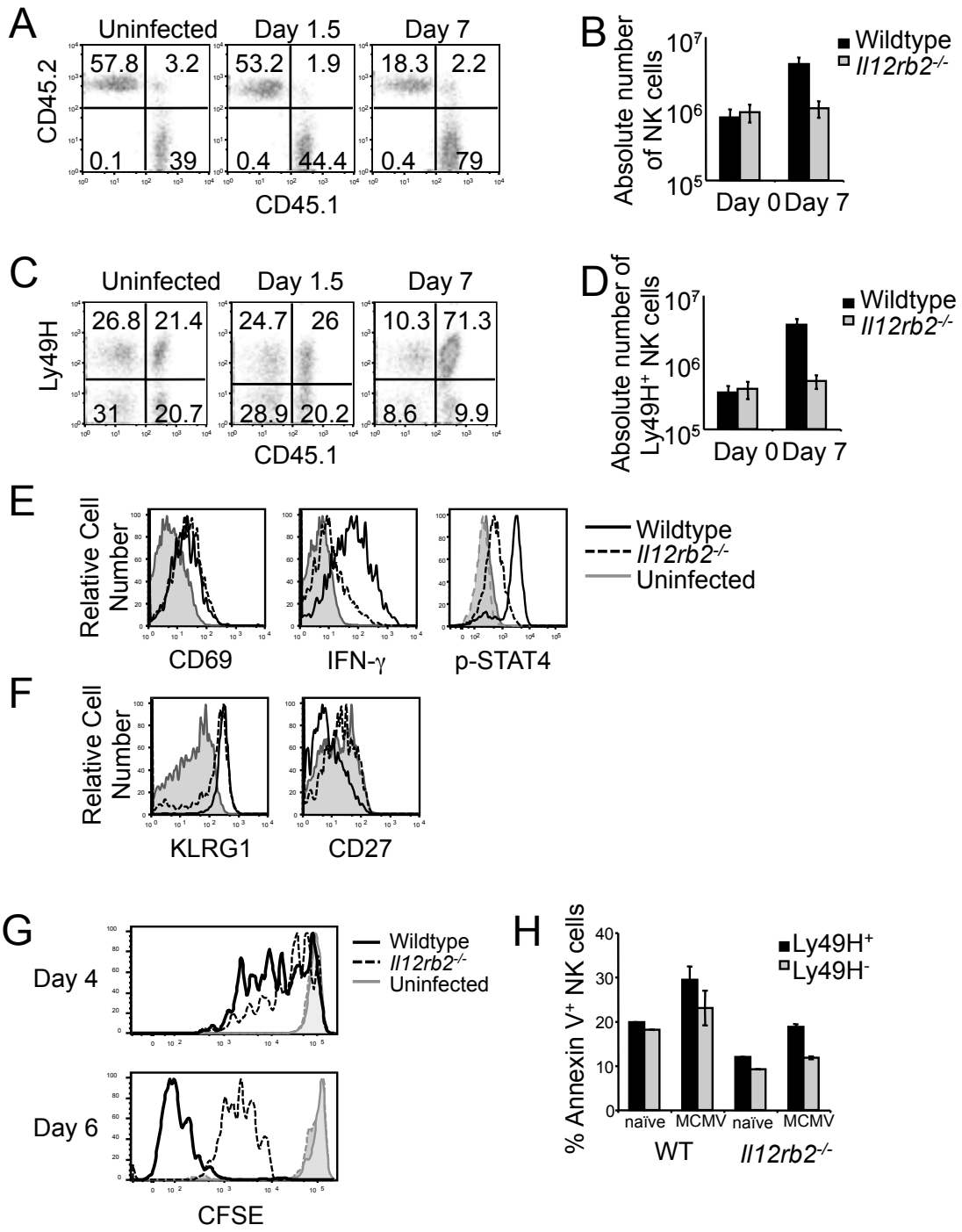

C
% CD107+ NK cells
100
75
50
25
0
Wildtype
Il12rb2-/-
No Ab
NK1.1
Ly49H
PMA/Ion


D
% killing
100
75
50
25
0
1:10
1:3
1:1
3:1
10:1
E:T ratio
wt
Il12rb2-/-
Ly49h-/-
+ BAF-m157
wt
Il12rb2-/-
Ly49h-/-
+ BAF

systems, uncontrolled global effects are expected due to the expression of the IL-12 receptor on many immune cell populations. Infection of IL-12-deficient mice results in higher viral titers (compared to wildtype mice) in many instances (72). To circumvent this problem, we generated mixed bone marrow chimeric mice, where approximately half of the hematopoietic compartment expressed the IL-12Rβ2 and the other half was deficient. Reconstitution of all immune cell populations was found to be equally distributed between WT and *Il12rb2*$^{-/-}$, including the NK cell compartment (Figure 2.2A, and data not shown).

Following MCMV infection of mixed chimeric mice, WT NK cells preferentially expanded over 7 days and became the predominant NK cell subset in the spleen (Figure 2.2A). A roughly 5-fold increase in total numbers of WT compared to *Il12rb2*$^{-/-}$ NK cells was observed at day 7 post-infection (PI) (Figure 2.2B). A similar outcome was observed in non-lymphoid organs such as the liver (data not shown). The expansion of the WT NK cell compartment was due to the rapid proliferation of Ly49H-bearing cells (Figure 2.2C). Although the uninfected chimeric mice contained similar numbers of WT and *Il12rb2*$^{-/-}$ Ly49H^{+} NK cells, by seven days PI the wildtype Ly49H^{+} NK cells were nearly 10-fold higher in absolute number compared to *Il12rb2*$^{-/-}$ Ly49H^{+} NK cells (Figure 2.2D). Consistent with a role for IL-12 in IFN-γ induction, fewer *Il12rb2*$^{-/-}$ NK cells produced IFN-γ compared with WT NK cells at day 1.5 PI, and the *Il12rb2*$^{-/-}$ NK cells made less IFN-γ per cell (as measured by mean fluorescence intensity). Interestingly, WT and *Il12rb2*$^{-/-}$ NK cells similarly upregulated the activation marker CD69 (Figure 2.2E). Induction of CD69 expression correlates with type I IFN-mediated activation, and

Figure 2.2. IL-12R-deficient NK cells exhibit defective proliferation during MCMV infection. (**A**) Mixed bone marrow chimeric mice were infected with MCMV and percentages of splenic WT ($CD45.1^+$) and *Il12rb2*$^{-/-}$ ($CD45.2^+$) NK cells are shown (gated on $CD3^-$ $NK1.1^+$) in uninfected mice and various time points post-infection. (**B**) The absolute numbers of splenic WT and *Il12rb2*$^{-/-}$ NK cells on day 0 and 7 PI are graphed. (**C**) Percentages of $Ly49H^+$ cells within the WT and *Il12rb2*$^{-/-}$ NK cell population (TCR-β^- $NK1.1^+$) are shown for uninfected mice and various time points post-infection. (**D**) The absolute numbers of WT and *Il12rb2*$^{-/-}$ $Ly49H^+$ NK cells on day 0 and 7 PI are graphed. (**E**) Expression of CD69, production of IFN-γ, and phosphorylation of STAT4 are shown for WT and *Il12rb2*$^{-/-}$ NK cells (compared to uninfected mice) at day 1.5 PI. (**F**) Expression of KLRG1, CD27, CD90 (Thy-1), Ly6C, and Ki67 on WT and *Il12rb2*$^{-/-}$ $Ly49H^+$ NK cells (compared to uninfected mice) at day 7 PI. (**G**) WT or *Il12rb2*$^{-/-}$ NK cells ($CD45.2^+$) were labeled with 5 μM CFSE and transferred into Ly49H-deficient hosts ($CD45.1^+$). Following MCMV infection, dividing NK cells were analyzed at day 4 and 6 PI (compared to uninfected control mice). (**H**) Adoptively transferred WT and *Il12rb2*$^{-/-}$ NK cells were stained for Annexin V at day 0 and 4 PI. Percentage of $Ly49H^+$ and $Ly49H^-$ NK cells positive for Annexin V are shown. Error bars for all graphs show s.e.m. (n = 3–5 for each time point) and all data are representative of 5 independent experiments.


A
Uninfected
Day 1.5
Day 7
CD45.2
57.8
3.2
53.2
1.9
18.3
2.2
0.1
39
0.4
44.4
0.4
79
CD45.1
B
Absolute number of NK cells
Wildtype
Il12rb2-/-
Day 0
Day 7
C
Ly49H
26.8
21.4
24.7
26
10.3
71.3
31
20.7
28.9
20.2
8.6
9.9
D
Absolute number of Ly49H+ NK cells
E
Relative Cell Number
CD69
IFN-γ
p-STAT4
Uninfected
F
KLRG1
CD27
G
Day 4
Day 6
CFSE
H
% Annexin V+ NK cells
Ly49H+
Ly49H-
naïve
MCMV
WT

our current data supports a mechanism whereby IFN-γ secretion and CD69 expression result from two segregated signaling pathways even though both are determinants of activation status. Phosphorylation of the signaling component STAT4 has been shown to be a consequence of IL-12R signaling leading to IFN-γ induction (71); thus, we examined splenic NK cells for phosphorylation of STAT4 early after infection. Whereas robust phosphorylation of STAT4 was observed in nearly all WT NK cells on day 1.5 PI, *Il12rb2*$^{-/-}$ NK cells showed minimal levels of phosphorylated STAT4 (Figure 2.2E). Nearly half of the *Il12rb2*$^{-/-}$ NK cells remained unphosphorylated for STAT4, at levels comparable to uninfected WT and *Il12rb2*$^{-/-}$ mice (Figure 2.2E). Splenic T cells from the same infected mice showed minimal amounts of STAT4 phosphorylation in both WT and *Il12rb2*$^{-/-}$ mice (data not shown), consistent with previous findings that NK cells are the early and major producers of IFN-γ during infection (77). At day 7 PI, WT Ly49H^{+} NK cells strongly upregulated KLRG1, Ly6C, CD90 (Thy-1), and Ki67 (a nuclear marker of cellular proliferation), and downregulated CD27, in contrast to *Il12rb2*$^{-/-}$ Ly49H^{+} NK cells (Figure 2.2F), suggesting that IL-12 signals during MCMV infection are crucial towards achieving a full maturation program in virus-specific NK cells.

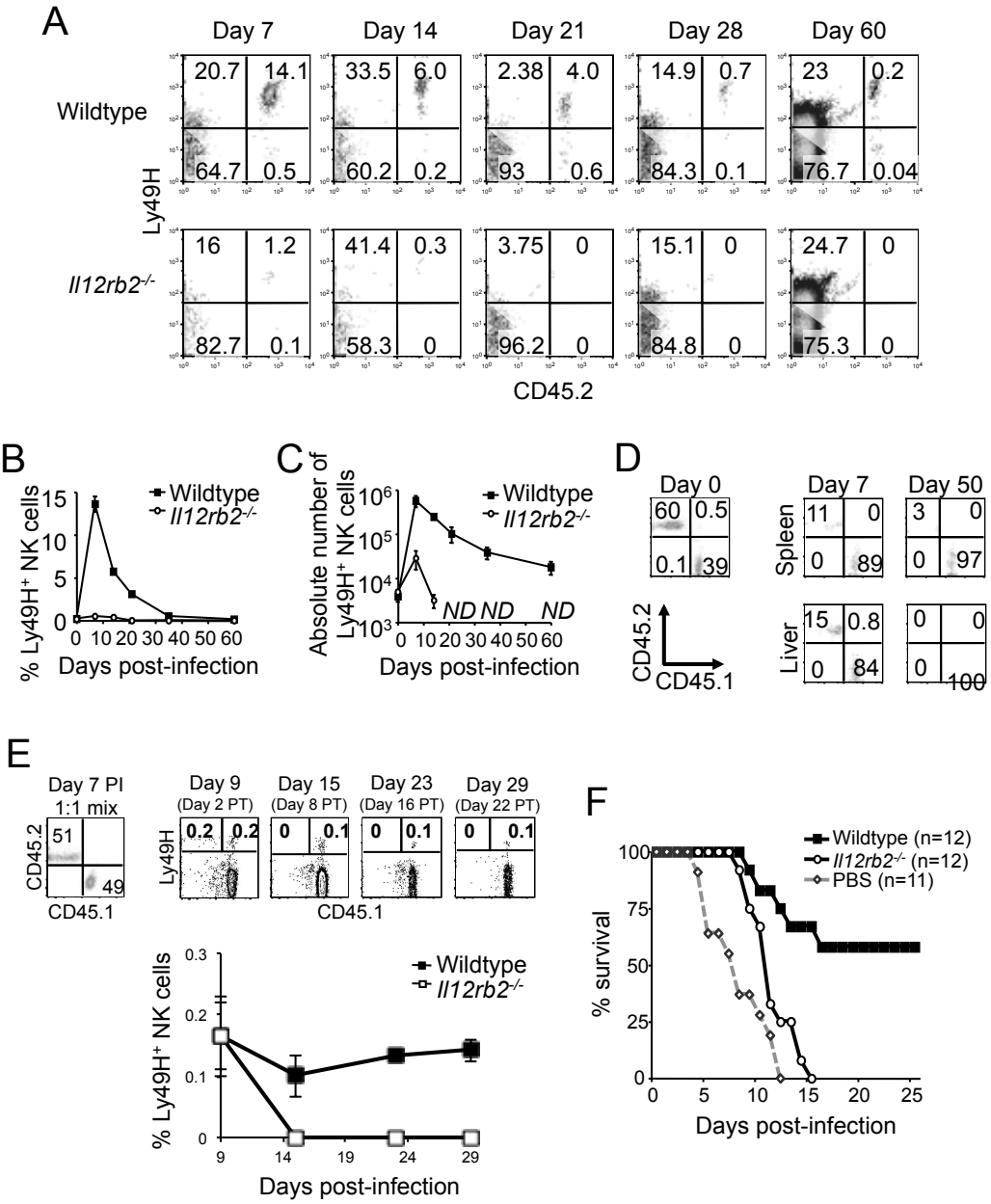
We determined whether the *Il12rb2*$^{-/-}$ NK cells were expanding as extensively as WT NK cells, or were dying faster once activated. Adoptive transfer of carboxyfluorescein succinimidyl ester (CFSE)-labeled WT or *Il12rb2*$^{-/-}$ NK cells into Ly49H-deficient mice confirmed that both WT and *Il12rb2*$^{-/-}$ NK cells proliferated after infection, with the WT NK cells dividing more extensively (Figure 2.2G). By day 6 PI, the WT NK cells had fully diluted their CFSE, whereas the

Il12rb2$^{-/-}$ NK cells remained intermediate for CFSE staining (Figure 2.2G); these results corroborate the higher expression of Ki67 detected on WT compared to *Il12rb2*$^{-/-}$ NK cells at day 7 PI (Figure 2.2F). At day 4 PI, WT and *Il12rb2*$^{-/-}$ NK cells showed comparable staining for Annexin V (Figure 2.2H), suggesting that a lack of IL-12 signal does not result in greater apoptosis.

IL-12 signals required for memory NK cell generation and protection during MCMV infection

A recent study demonstrated that although defective IL-12 signaling in $CD8^+$ T cells also resulted in fewer effector cells, surprisingly higher numbers of memory cells were generated (78). Therefore, we investigated the long-term consequences of IL-12 signaling during the NK cell response against MCMV infection. Using a previously described adoptive transfer system (13), we purified NK cells from WT and *Il12rb2*$^{-/-}$ mice and transferred an equal number of $Ly49H^+$ NK cells from each group into separate DAP12-deficient hosts, which are deficient in Ly49H-expressing NK cells. Following MCMV infection, we found that WT NK cells proliferated robustly in the new hosts, in contrast to *Il12rb2*$^{-/-}$ NK cells (Figure 2.3A). The percentage and absolute number of WT $Ly49H^+$ NK cells at day 7 PI were 15- to 20-fold higher than the *Il12rb2*$^{-/-}$ NK cells (Fig. 2.3a and b). Strikingly, whereas WT NK cells were easily recovered from recipient mice several weeks later, adoptively transferred *Il12rb2*$^{-/-}$ NK cells were not detectable after week 2 PI (Figure 2.3B). Co-adoptive transfer of equal numbers of WT and *Il12rb2*$^{-/-}$ NK cells into recipient mice yielded the same outcome following infection, with WT NK

Figure 2.3. NK cells from *Il12rb2*$^{-/-}$ mice fail to become long-lived memory cells and mediate protection following MCMV infection. (**A-B**) A total of 1x10^5 WT or *Il12rb2*$^{-/-}$ Ly49H$^+$ NK cells (both CD45.2$^+$) were transferred into DAP12-deficient mice (CD45.1$^+$) and infected with MCMV. (**A**) Plots are gated on total NK cells and percentages of adoptively transferred CD45.2$^+$ Ly49H$^+$ NK cells are shown for each time point PI. (**B**) Absolute number of adoptively transferred WT and *Il12rb2*$^{-/-}$ Ly49H$^+$ NK cells in the spleen of recipient mice are shown. Error bars show s.e.m. (*n* = 3–5). *ND*, not detectable (or below the limits of detection). (**C**) WT (CD45.1$^+$) and *Il12rb2*$^{-/-}$ (CD45.2$^+$) Ly49H$^+$ NK cells were co-adoptively transferred into Ly49H-deficient mice and infected with MCMV. Plots are gated on transferred NK cells and percentages of WT and *Il12rb2*$^{-/-}$ NK cells are shown for spleen and liver at day 0, 7, and 50 PI. All data are representative of five experiments with 3-5 mice per time point. (**D**) WT (CD45.1$^+$) and *Il12rb2*$^{-/-}$ (CD45.2$^+$) mice were infected with MCMV and splenic Ly49H$^+$ NK cells on day 7 PI were purified, mixed at a 1:1 ratio, and co-transferred into Ly49H-deficient mice. Plots are gated on transferred NK cells and percentages of WT and *Il12rb2*$^{-/-}$ NK cells are shown for at various time points PI and post-transfer (PT). All data are representative of three experiments with 2-4 mice per time point. The graph shows the percentage of adoptively transferred WT and *Il12rb2*$^{-/-}$ Ly49H$^+$ NK cells within the total NK cell population, and error bars show s.e.m. (*n* = 3–4). (**E**) DAP12-deficient neonatal mice received 1x10^5 WT or *Il12rb2*$^{-/-}$ NK cells (or PBS as control) followed by MCMV infection. The graph shows the percentage of surviving mice for each group, and data were pooled from three experiments.


A
Day 7
Day 14
Day 21
Day 28
Day 60
Wildtype
Il12rb2-/-
Ly49H
CD45.2
20.7 14.1 64.7 0.5
33.5 6.0 60.2 0.2
2.38 4.0 93 0.6
14.9 0.7 84.3 0.1
23 0.2 76.7 0.04
16 1.2 82.7 0.1
41.4 0.3 58.3 0
3.75 0 96.2 0
15.1 0 84.8 0
24.7 0 75.3 0
B
% Ly49H+ NK cells
Days post-infection
Wildtype
Il12rb2-/-
C
Absolute number of Ly49H+ NK cells
ND ND ND
Days post-infection
D
Day 0
Day 7
Day 50
Spleen
Liver
CD45.2
CD45.1
60 0.5 0.1 39
11 0 0 89
3 0 0 97
15 0.8 0 84
0 0 0 100
E
Day 7 PI
1:1 mix
Day 9
(Day 2 PT)
Day 15
(Day 8 PT)
Day 23
(Day 16 PT)
Day 29
(Day 22 PT)
CD45.2
CD45.1
Ly49H
51 49
0.2 0.2
0 0.1
0 0.1
0 0.1
% Ly49H+ NK cells
Days post-infection
Wildtype
Il12rb2-/-
F
% survival
Days post-infection
Wildtype (n=12)
Il12rb2-/- (n=12)
PBS (n=11)

cells preferentially expanding at day 7 PI to become the only memory NK cells detected months later in both spleen and liver (Figure 2.3C). To determine whether fewer *Il12rb2*$^{-/-}$ NK cells were recovered at later time point simply because they could not expand as well, equal numbers of WT and *Il12rb2*$^{-/-}$ effector Ly49H^{+} NK cells isolated at day 7 PI (1:1 mix) were adoptively transferred and memory cell percentages determined at later time points (Figure 2.3D). Although the day 7 effector NK cell numbers were experimentally normalized, only WT NK cells were detected one month later in recipient mice (Figure 2.3D). Thus, IL-12 signals are crucial not only for the optimal expansion of virus-specific NK cells during infection, but also for the generation of a long-lived NK cell population in lymphoid and non-lymphoid tissues.

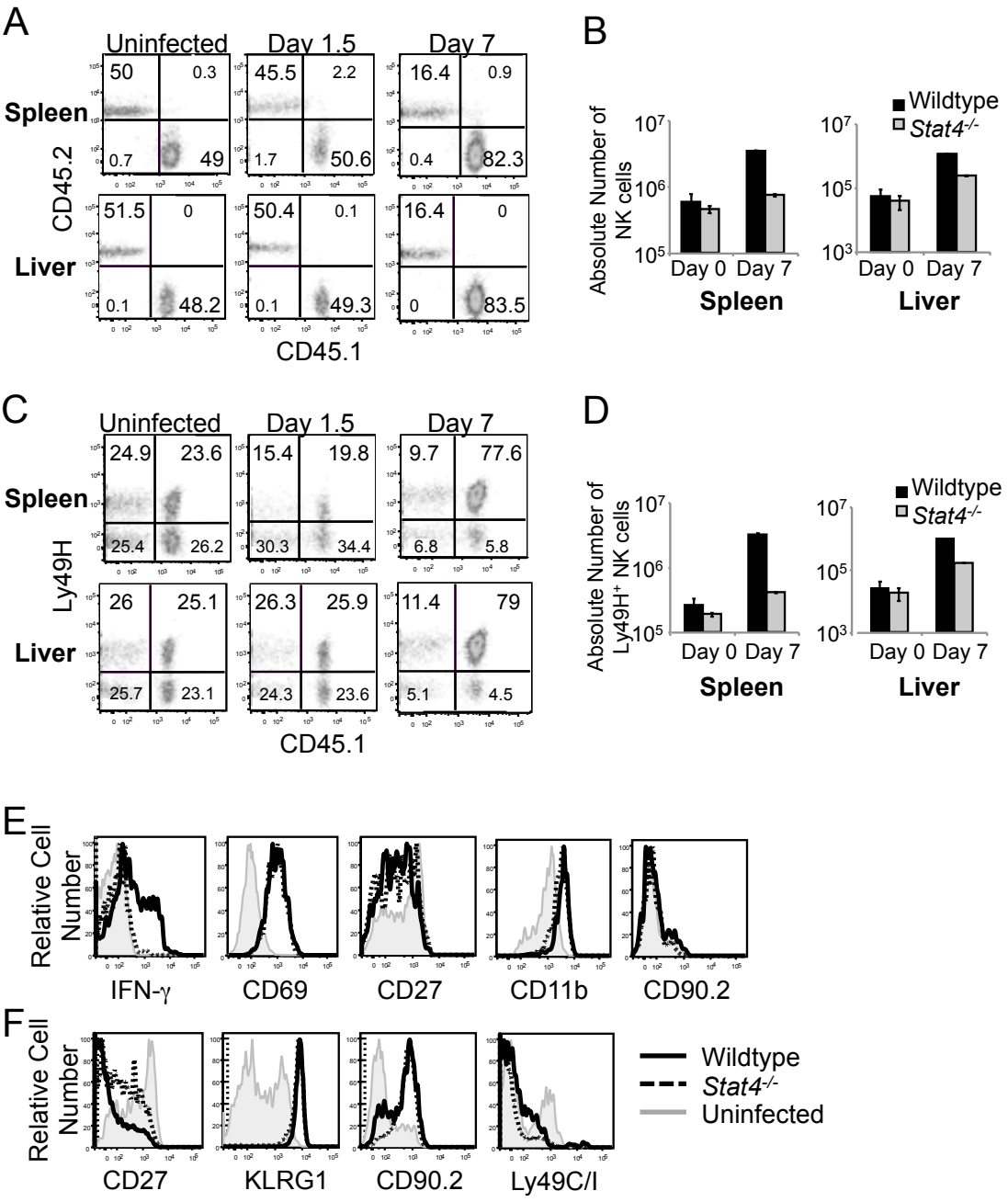
Because of the pleiotrophic effects complicating the direct infection of cytokine-deficient mice, it is unknown whether defective IL-12 signaling in NK cells alone will influence the course of viral infection (without loss of the IL-12R on other cell types). Thus, we examined the contribution of IL-12R signaling specifically in NK cells towards protection against MCMV infection. We transferred an equal number of purified NK cells from WT or *Il12rb2*$^{-/-}$ mice into neonatal DAP12-deficient mice. As a negative control, one group of neonates was given PBS without cells. All three groups were challenged with MCMV. Within the first 2 weeks, all of the mice receiving PBS or purified *Il12rb2*$^{-/-}$ NK cells had died (Figure 2.3E). During the same period, greater than 50% of the mice given purified WT NK cells survived (Figure 2.3E). Together, the data suggest that IL-12 signaling in NK cells alone is crucial for protective immunity against viral challenge.

Interestingly, the mice receiving *Il12rb2*$^{-/-}$ NK cells died with delayed kinetics (days 9-15 PI) compared to mice receiving PBS (days 5-12 PI), perhaps because the *Il12rb2*$^{-/-}$ NK cells could still mediate cytotoxicity against infected cells; however, their inability to undergo proliferation similar to WT NK cells might have prevented a more efficacious response.

NK cell memory depends on STAT4 signals, but not IFN-γ secretion

We investigated the importance of effector molecules downstream of the IL-12R in the generation of long-lived memory NK cells during MCMV infection. Because *Il12rb2*$^{-/-}$ NK cells do not efficiently phosphorylate STAT4 or secrete as much IFN-γ as WT NK cells during MCMV infection (Figure 2.2E), we examined the contribution of both STAT4 and IFN-γ on the production of effector and memory Ly49H^{+} NK cells. To elucidate the importance of downstream effector molecule STAT4 on *in vivo* NK cell activation and response against MCMV, we generated mixed bone marrow chimeric mice. Reconstitution of all immune populations, including the NK compartment, was found to be equally distributed between WT and *Stat4*$^{-/-}$ (Figure 2.4A and data not shown). Following MCMV infection, wildtype NK cells preferentially expanded over 7 days to constitute the main subset in the spleen and liver, exhibiting 5-fold greater numbers compared to *Stat4*$^{-/-}$ NK cells (Figure 2.4A-B), similar to that seen for mixed WT:*Il12rb2*$^{-/-}$ bone marrow chimeras (Figure 2.2A). In both organs, the prolific expansion of WT NK cells can be attributed to the proliferation of Ly49H-bearing cells (Figure 2.4C). Whereas uninfected mice had comparable numbers of WT and *Stat4*$^{-/-}$ NK cells, by day 7 PI

Figure 2.4. Wildtype NK cells out-compete *Stat4*$^{-/-}$ NK cells during MCMV infection. (**A**) Mixed bone marrow chimeric mice were infected with MCMV and percentages of WT (CD45.1^{+}) and *Stat4*$^{-/-}$ (CD45.2^{+}) NK cells in spleen and liver are shown for uninfected mice and various time points PI. (**B**) The graph shows absolute numbers of WT and *Stat4*$^{-/-}$ NK cells in spleen and liver on day 0 and 7 PI. Error bars show s.e.m. (n = 3–5). (**C**) Percentages of Ly49H^{+} cells within the WT and *Stat4*$^{-/-}$ NK cell population in spleen and liver are shown for uninfected mice and various time points PI. (**D**) The graph shows absolute numbers of WT and *Stat4*$^{-/-}$ Ly49H^{+} NK cells in spleen and liver on day 0 and 7 PI. Error bars show s.e.m. (n = 3–5). (**E**) Expression of CD69, CD27, CD11b, and CD90, and production of IFN-γ are shown for WT and *Stat4*$^{-/-}$ NK cells (compared to uninfected mice) at day 1.5 PI. (**F**) Expression of KLRG1, CD90, Ly49C/I, and CD27 are shown for WT and *Stat4*$^{-/-}$ Ly49H^{+} NK cells (compared to uninfected mice) at day 7 PI. All data are representative of three experiments with 3-5 mice per time point.


A
Uninfected
Day 1.5
Day 7
Spleen
Liver
CD45.2
CD45.1
50
0.3
0.7
49
45.5
2.2
1.7
50.6
16.4
0.9
0.4
82.3
51.5
0
0.1
48.2
50.4
0.1
0.1
49.3
16.4
0
0
83.5
B
Absolute Number of NK cells
Wildtype
Stat4-/-
Day 0
Day 7
Spleen
Liver
C
Uninfected
Day 1.5
Day 7
Spleen
Liver
Ly49H
CD45.1
24.9
23.6
25.4
26.2
15.4
19.8
30.3
34.4
9.7
77.6
6.8
5.8
26
25.1
25.7
23.1
26.3
25.9
24.3
23.6
11.4
79
5.1
4.5
D
Absolute Number of Ly49H+ NK cells
Wildtype
Stat4-/-
Day 0
Day 7
Spleen
Liver
E
Relative Cell Number
IFN-γ
CD69
CD27
CD11b
CD90.2
F
Relative Cell Number
CD27
KLRG1
CD90.2
Ly49C/I
Wildtype
Stat4-/-
Uninfected

the WT Ly49H$^+$ NK cells outnumbered their *Stat4*$^{-/-}$ counterparts in absolute numbers by approximately 10-fold in spleen and liver (Figure 2.4D). Early after infection, *Stat4*$^{-/-}$ NK cells produced less IFN-γ than WT NK cells, even though CD69 was similarly upregulated in both subsets at day 1.5 PI (Figure 2.4E). Although WT and *Stat4*$^{-/-}$ NK cells similarly downregulated CD27 and upregulated CD11b at day 1.5 PI, phenotypic differences were evident at day 7 PI in spleen and liver, with WT NK cells strongly downregulating CD27 and upregulating KLRG1 (Figure 2.4E). Together, these findings suggest that IL-12-induced signaling in virus-specific NK cells primarily uses the downstream STAT4 to initiate vital signals for a complete cell maturation program during MCMV infection. Interestingly, both WT and *Stat4*$^{-/-}$ NK cells at day 7 PI highly expressed CD90 (Thy-1) (Figure 2.4F), a marker described to be present on memory NK cells (79, 80), and were predominantly Ly49C/I$^-$ (Figure 2.4F), consistent with previous data demonstrating that Ly49C/I$^-$ or "unlicensed" NK cells in C57BL/6 mice dominate the NK cell response to MCMV (81).

We investigated the extent to which signaling through STAT4 influenced the generation of memory NK cells during MCMV infection. We transferred an equal number of Ly49H$^+$ NK cells purified from WT (CD45.1) and *Stat4*$^{-/-}$ (CD45.2) mice into a Ly49H-deficient host (CD45.2). Transferred WT Ly49H$^+$ NK cells proliferated robustly following MCMV infection, in contrast to the minimal expansion of transferred *Stat4*$^{-/-}$ Ly49H$^+$ NK cells in the same host (Figure 2.5A). The percentage of WT NK cells at day 7 PI was approximately 10-fold higher than that of the co-transferred *Stat4*$^{-/-}$ counterparts (Figure 2.5B), highlighting the

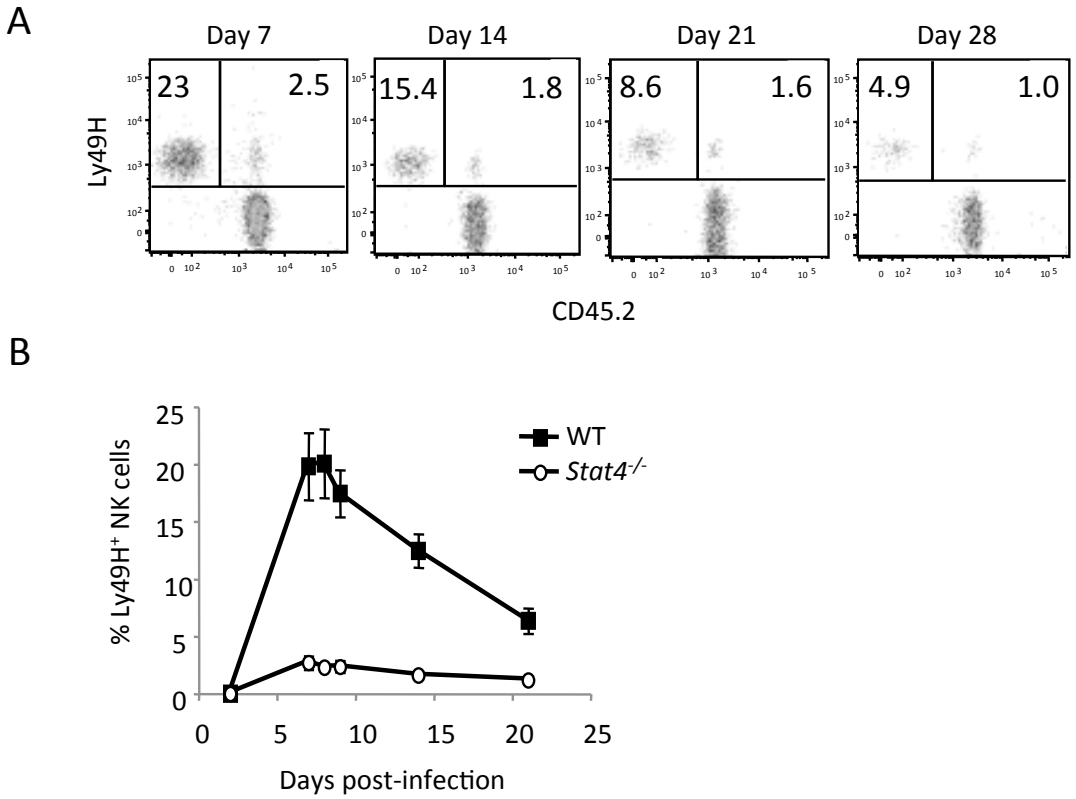
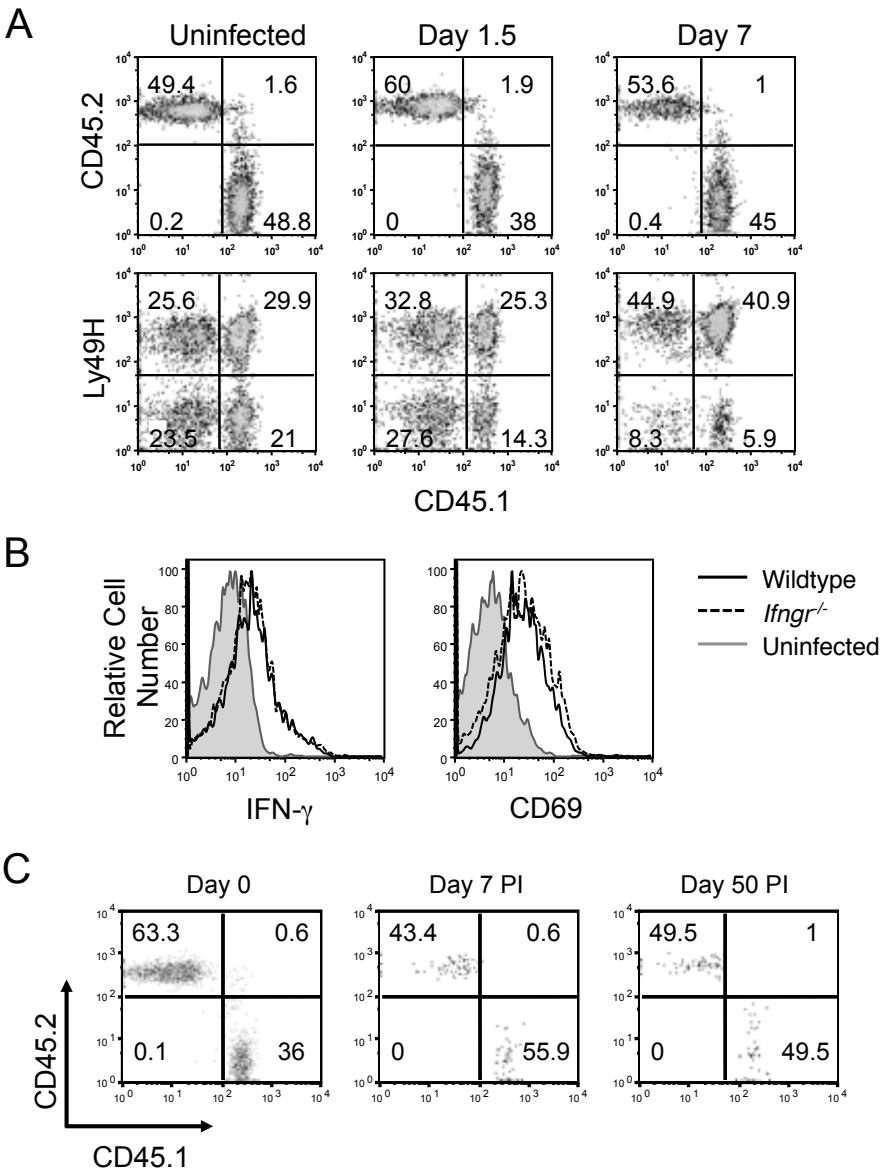


Figure 2.5. Defective memory NK cell generation in the absence of STAT4. (**A**) A total of 1x10^5 WT (CD45.1$^+$) and *Stat4*$^{-/-}$ (CD45.2$^+$) Ly49H$^+$ NK cells were co-transferred into Ly49H-deficient mice (CD45.2$^+$) and infected with MCMV. Plots are gated on total NK cells and percentages of adoptively transferred Ly49H$^+$ NK cells (wildtype in upper left quadrant and *Stat4*$^{-/-}$ in upper right quadrant) are shown for each time point PI. (**B**) Percentage of adoptively transferred WT and *Stat4*$^{-/-}$ Ly49H$^+$ NK cells within the total NK cell population are shown. Error bars show s.e.m. (*n* = 3). All data are representative of three experiments with 3-4 mice per time point.

importance of signaling through STAT4 in the expansion of virus-specific NK cells following MCMV infection and in the attainment of a long-lived memory NK cell population.

Lastly, we examined a possible autocrine role for IFN-γ produced by NK cells during infection. Because WT NK cells produced more IFN-γ than *Il12rb2*$^{-/-}$ or *Stat4*$^{-/-}$ NK cells early during MCMV infection, could this IFN-γ "feedback" on the NK cells themselves and drive stronger proliferation and generation of memory cells? We first generated mixed bone marrow chimeric mice consisting of a 1:1 ratio of WT:*Ifngr*$^{-/-}$ hematopoietic cells. Following MCMV infection of chimeric mice, WT and *Ifngr*$^{-/-}$ NK cells proliferated similarly over the first 7 days (Figure 2.6A), with nearly equal ratio of WT to *Ifngr*$^{-/-}$ NK cells. On day 7 PI, similar percentages of Ly49H-bearing cells existed in total WT and *Ifngr*$^{-/-}$ NK cell populations (Figure 2.6A), suggesting that IFN-γ does not influence the ability of NK cells themselves to undergo expansion. On day 1.5 PI, expression of CD69 and secretion of IFN-γ were also independent of IFN-γ receptor expression on the NK cell (Figure 2.6B). Similarly, no phenotypic differences were observed between WT and *Ifngr*$^{-/-}$ NK cells at day 7 PI (data not shown). When we co-transferred equal numbers of enriched WT and *Ifngr*$^{-/-}$ NK cells into recipient mice followed by MCMV infection, we observed a similar expansion at day 7 PI and equal percentages of memory NK cells at day 50 PI (Figure 2.6C). Altogether, these data suggest that the robust generation of NK cell memory during MCMV infection requires STAT4-dependent, but IFN-γ independent signals.

Figure 2.6. Wildtype and *Ifngr*$^{-/-}$ NK cells expand equally and generate memory cells following MCMV infection. (**A**) Mixed-bone-marrow chimeric mice consisting of a 1:1 mixture of WT (CD45.1^{+}) and *Ifngr*$^{-/-}$ (CD45.2^{+}) hematopoietic cells were infected with MCMV. Top row: percentages of total WT and *Ifngr*$^{-/-}$ NK cells (gated on CD3^{-} NK1.1^{+}) are shown for uninfected mice and at day 1.5 and 7 PI. Bottom row: percentages of Ly49H^{+} cells within the WT and *Ifngr*$^{-/-}$ NK cell populations are shown. (**B**) Production of IFN-γ and expression of CD69 are shown for WT and *Ifngr*$^{-/-}$ NK cells in chimeric mice (compared to uninfected chimeras) at day 1.5 PI. (**C**) WT (CD45.1^{+}) and *Ifngr*$^{-/-}$ (CD45.2^{+}) Ly49H^{+} NK cells were co-adoptively transferred into Ly49H-deficient mice and infected with MCMV. Plots are gated on transferred NK cells and percentages of WT and *Ifngr*$^{-/-}$ NK cells are shown for day 0, 7, and 50 PI. All data are representative of three experiments with 3-5 mice per time point.


A
Uninfected
Day 1.5
Day 7
CD45.2
Ly49H
CD45.1
49.4
1.6
0.2
48.8
60
1.9
0
38
53.6
1
0.4
45
25.6
29.9
23.5
21
32.8
25.3
27.6
14.3
44.9
40.9
8.3
5.9
B
Relative Cell Number
IFN-γ
CD69
Wildtype
Ifngr-/-
Uninfected
C
Day 0
Day 7 PI
Day 50 PI
CD45.2
CD45.1
63.3
0.6
0.1
36
43.4
0.6
0
55.9
49.5
1
0
49.5

The recent discovery that primed NK cells can become long-lived memory cells during infection begs the question of what the mechanisms are behind such a phenomena. Past studies using neutralizing antibodies and cytokine deficient mice did not specifically address whether there was an *in vivo* role for IL-12 on NK cells independent of global effects on other cell types (29, 72-76). Although these previous studies found a role for IL-12 in activating NK cells, our current study demonstrates the absolute requirement for both IL-12 and STAT4 in the clonal expansion of antigen-specific NK cells and the generation of memory NK cells during MCMV infection.

The amount of inflammation in the host environment during $CD8^+$ T cell priming has been suggested to dictate the production of effector and memory cells. In recent studies where the degree of inflammation was varied while antigen concentration was kept constant, memory $CD8^+$ T cell potential was determined by a gradient of T-bet expression in which moderate inflammation correlated with robust memory cells, whereas high inflammation correlated with short-lived effector cells and reduced memory potential (24, 82). Consistent with this study, another group showed that IL-12-deficient mice produced a diminished effector $CD8^+$ T cell response during Listeria infection, but generated higher numbers of memory $CD8^+$ T cells and greater protection against re-infection (78). However, when mice receiving wildtype or IL-12R-deficient TCR transgenic $CD8^+$ T cells were infected with viral pathogens, the conclusions were less clear. One study observed normal clonal expansion but diminished memory responses in $CD8^+$ T cells that could not sense IL-12 during vaccinia virus infection (83), whereas another study showed

normal effector and memory responses during infection with vaccinia virus, LCMV, and VSV (84). These discrepancies may be due to different TCR transgenic mice and recombinant pathogens used in the studies, but highlight the need to resolve these inconsistencies.

In contrast to the inconclusive role of IL-12 on T cells, the NK cell response in the absence of IL-12 signaling resulted in a severe detriment in both effector and memory NK cell numbers. During MCMV infection, the IL-12R-deficient Ly49H$^+$ NK cells showed a remarkable 10- to 20-fold reduction in absolute number of effector cells in spleen and liver compared to WT NK cells, and a complete deficiency in memory cells. Even when effector NK cell numbers (isolated at day 7 PI) were normalized, only WT NK cells could be detected at later time points following adoptive transfer (Figure 2.3D), suggesting that IL-12 signaling "programs" NK cells during priming to demonstrate longevity following activation and expansion. Unlike CD8$^+$ T cells that expanded less in the absence of IL-12 but generated higher memory cell numbers (78), our results suggest an absolute requirement for IL-12 signals in the generation of memory NK cells. These findings are strengthened by the severe defect in clonal expansion and memory cell formation in STAT4-deficient NK cells. Interestingly, we do observe some low level phosphorylation of STAT4 in the *Il12rb2*$^{-/-}$ NK cells compared to WT NK cells (Figure 2.2E), suggesting that other cytokines produced during MCMV infection may share the STAT4 signaling pathway. IFN-α/β has been shown to activate STAT4 (in addition to STAT1) in T cells (40); however, findings in IFN-α receptor-deficient mice that STAT4 is normally phosphorylated during MCMV

infection suggest that IL-12 signaling is the predominant pathway for STAT4 phosphorylation leading to IFN-γ production in NK cells (40) (Firth and Sun, unpublished observations). Developing a system for NK cells where we can vary the inflammation while keeping the antigen dose constant will allow us to determine whether, like $CD8^+$ T cells, there exist optimal and sub-optimal degrees of inflammation that drive generation of effector and memory NK cells. In addition, the molecular mechanisms behind NK cell programming by IL-12 during MCMV infection remain to be elucidated. Future studies will determine how IL-12 signaling influences histone acetylation and chromatin remodeling at promoter sites of genes important for the generation of NK cell memory.

Like NK cells, memory $CD8^+$ T cells surprisingly possess the "innate-like" ability to produce IFN-γ and proliferate following exposure to pro-inflammatory cytokines alone, without TCR and costimulatory receptor ligation (85). Given their relatedness during ontogeny (both are derived from a common lymphoid progenitor) and possible shared evolutionary ancestry, perhaps it is not surprising that parallel mechanisms exist in NK cells and T cells for their activation, proliferation, and generation of memory during infection. The importance of environmental cues, in addition to antigen receptor triggering, is becoming more evident, and a greater understanding of these mechanisms will allow for development of therapeutics and vaccines against infectious disease.

Materials and Methods

Mice and infections.

C57BL/6 and congenic CD45.1^{+} mice were purchased from the National Cancer Institute. IL-12 receptor β2 chain-deficient (75), STAT4-deficient (86), IFN-γ receptor-deficient, β2m-deficient, Ly49H-deficient (87), and DAP12-deficient (88) mice on a C57BL/6 background were bred at UCSF and MSKCC and maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee. Mixed-bone-marrow chimeric mice were generated as described previously (13). Mice were infected by intraperitoneal injections of Smith strain MCMV (5×10^4 plaque-forming units (p.f.u.)). Newborn mice were infected with 2×10^3 p.f.u. of MCMV.

Flow cytometry.

Fc receptors were blocked with 2.4G2 mAb before surface or intracellular staining with indicated antibodies or isotype-matched control immunoglobulin (BD, eBioscience, or BioLegend). For measuring apoptosis, freshly isolated splenocytes were first stained with antibodies against NK1.1, CD3, and Ly49H, and a Live/Dead fixable near-IR stain (Invitrogen), washed, and then stained with PE-conjugated Annexin V (BD), according to manufacture's protocol. Samples were acquired on an LSRII (BD) and analyzed with FlowJo software (TreeStar).

NK cell enrichment, CFSE-labeling, and adoptive transfer.

NK cells were enriched with an NK-cell Isolation Kit (Miltenyi Biotec) or a method where spleen cells were incubated with purified rat antibodies against mouse CD4, CD5, CD8, CD19, Gr-1, and Ter119 followed by anti-rat IgG antibodies conjugated to magnetic beads (Qiagen). Purified NK cells were injected intravenously into adult recipients or intraperitoneally into neonatal recipients one day before viral infection. In some experiments, NK cells or unfractionated splenocytes were labeled with varying concentrations of CFSE prior to intravenous injection. Labeling of cells with CFSE was performed in accordance with the manufacturer's instructions (Invitrogen).

Ex vivo stimulation of NK cells and cytotoxicity assay.

Tissue culture plates treated with *N*-(1-(2,3-dioleoyloxyl)propyl)-*N*,*N*,*N*-trimethylammonium methylsulphate (Sigma) were coated with anti-NK1.1 or anti-Ly49H antibodies (10 μg/ml) and enriched NK cells or whole splenocytes were incubated for 5 h at 37 °C in the presence of Golgiplug (BD), followed by staining for intracellular cytokines. Uncoated or PBS-coated wells served as negative controls, and addition of PMA (50 ng/mL) and Ionomycin (1 μg/mL) during incubation served as a positive control.

Enriched NK cells were used as effector cells *ex vivo* in a 6-h ^{51}Cr release assay against Ba/F3 and m157-transfected Ba/F3 target cells. Percentage of Ly49H^{+} NK cells in each group was determined by flow cytometry and absolute numbers were normalized prior to incubation with targets. Large numbers of NK cells from

Ly49H-deficient mice were included as a negative control to demonstrate specificity of receptor-ligand interactions.

Statistical methods.

The Mann–Whitney nonparametric *U*-test was used to compare survival between groups of mice. A value of 25 days was assigned to survivors living more than 25 days after MCMV infection. Student's *t*-test was used to compare groups in *ex vivo* stimulation experiments.

CHAPTER 3

STAGE-SPECIFIC REQUIREMENT OF IL-18 FOR ANTIVIRAL NK CELL EXPANSION

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Introduction

Natural Killer (NK) cells play a significant role in the control of infected, stressed, or transformed cells that may be detrimental to the host. Recent studies in mice and humans have demonstrated that NK cells possess adaptive immune qualities (89). In mice infected with mouse cytomegalovirus (MCMV), $Ly49H^+$ NK cells activated by the viral glycoprotein m157 undergo extensive proliferation, and contract resulting in the formation of a small pool of long-lived memory NK cells (13) that can be recalled, and exhibit heightened effector function (69, 89).

Pro-inflammatory cytokines strongly influence the NK cell response against MCMV infection (90). Although previous work has described the effect of pro-inflammatory cytokines on the general activation of NK cells during MCMV infection (90), their role in driving clonal-like expansion and memory in antigen-specific NK cells is largely unknown. We previously implicated IL-12, its signaling molecule STAT4, and the downstream transcription factor Zbtb32 as crucial signals in the generation of robust effector and memory NK cell responses against MCMV infection (91, 92). IL-18 has been suggested to "prime" resting NK cells for

maximum IFN-γ production following *ex vivo* stimulation (48), and synergize with IL-12 during NK cell activation (93). Although IL-18 is produced early during MCMV infection (31), it is not known how IL-18 signals influence the virus-specific Ly49H$^+$ NK cell response. Here, we investigate the direct effects of IL-18 signaling on primary and recall NK cell responses to MCMV infection.

Results and Discussion

Cell-Intrinsic IL-18 signaling required for the primary expansion of virus-specific NK cells

Although IL-18 has previously been shown to activate NK cells during viral infection, these studies involve directly infecting IL-18- and IL-18 receptor-deficient mice (31, 32). Therefore, to address whether IL-18 influences NK cell responses in a cell-intrinsic manner, we adoptively transferred equal numbers of WT and *Il18r1*$^{-/-}$ NK cells into *Ly49h*$^{-/-}$ mice, which harbor normal numbers of NK cells but are incapable of recognizing the MCMV-derived m157 protein (13, 91). Following infection with MCMV, WT NK cells preferentially expanded during the first week of infection and were higher in frequency than *Il18r1*$^{-/-}$ NK cells at day 7 post-infection (PI; Supplementary Figure 3.1A) and at later time points (Figure 3.1A). Consistent with the adoptive transfer experiment, we observed a similar expansion defect by *Il18r1*$^{-/-}$ Ly49H$^+$ NK cells in WT:*Il18r1*$^{-/-}$ mixed bone marrow chimeric mice infected with MCMV (Figure 3.1B and Supplementary Figure 3.1B). Together, these studies confirm a cell-intrinsic requirement for IL-18 signaling in the antiviral NK cell response.

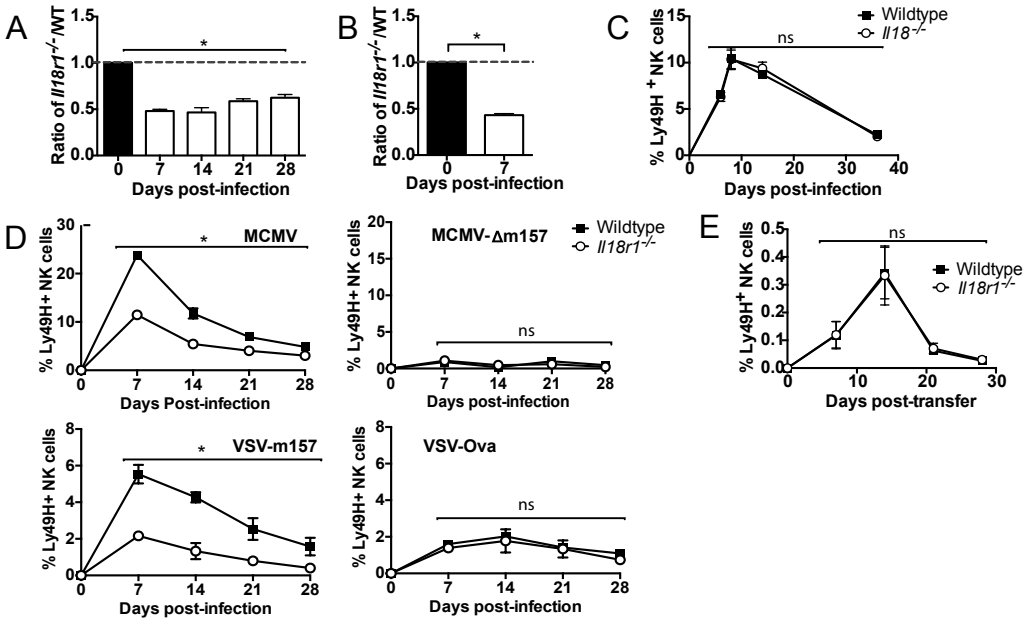


Figure 3.1. IL-18R-deficient NK cells mount a defective response to viral infection. A. WT and *Il18r1*$^{-/-}$ NK cells were co-transferred into *Ly49h*$^{-/-}$ mice and infected with MCMV. The relative ratio of populations is shown for each time point compared to day 0. **B.** Mixed WT:*Il18r1*$^{-/-}$ chimeric mice were infected with MCMV and the relative ratio of Ly49H^{+} NK cells are shown for day 7 PI compared to uninfected. **C.** Percentages of co-transferred WT and *Il18*$^{-/-}$ Ly49H^{+} NK cells are shown during MCMV infection. **D.** Percentages of co-transferred WT and *Il18r1*$^{-/-}$ Ly49H^{+} NK cells are shown following infection with MCMV, MCMV-Δm157, VSV-m157, or VSV-Ova. **E.** WT and *Il18r1*$^{-/-}$ NK cells were co-transferred into *Rag x Il2rg*$^{-/-}$ mice and percentages of transferred Ly49H^{+} NK cells within the total cell population are shown. Data are mean ± s.e.m. representative of at least four independent experiments with at least n=3 biological replicates per condition. * $p <$ 0.05 and ns, not significant, paired Student *t*-test.

IL-18 has been suggested enhance IL-12-induced effector functions of NK cells such as IFN-γ production (48, 93). To determine if IL-18 might also "prime" NK cells for MCMV-driven expansion, we isolated resting NK cells from *Il18*$^{-/-}$ mice and co-transferred them with equal numbers of WT NK cells into *Ly49h*$^{-/-}$ hosts. Following MCMV infection, WT and *Il18*$^{-/-}$ Ly49H^{+} NK cells exhibited comparable expansion and memory cell formation (Figure 3.1C), indicating that previous exposure to IL-18 during development or homeostasis was not required for normal expansion in response to viral challenge as long as IL-18 is present during infection.

To determine whether the effect of IL-18 signaling was limited to antigen-specific NK cell responses, we utilized MCMV and VSV viruses engineered to lack or express m157, respectively. Following adoptive transfer of WT and *Il18r1*$^{-/-}$ NK cells into *Ly49h*$^{-/-}$ hosts, we observed that in contrast to MCMV, infection with MCMV lacking m157 (MCMV-Δm157) elicited an equivalent (albeit modest) expansion of both WT and *Il18r1*$^{-/-}$ NK cell populations (Figure 3.1D), indicating that IL-18 signaling is not required for the low-level proliferation of "bystander" NK cells responding to viral infection. Consistent with this observation, infection with VSV-m157, but not with VSV-Ova, elicited a preferential antigen-specific expansion of WT NK cells over *Il18r1*$^{-/-}$ NK cells (Figure 3.1D). We next investigated whether IL-18 was required for NK cells to undergo homeostatic proliferation. We found that WT and *Il18r1*$^{-/-}$ NK cells proliferated similarly when transferred into *Rag2*$^{-/-}$ *x Il2rg*$^{-/-}$ mice, generating comparable long-lived populations more than 4 weeks later (Figure 3.1E), demonstrating IL-18 signaling is not required

for NK cells to proliferate in response to common gamma chain cytokines. Altogether, these data suggest that IL-18 signaling directly regulates antigen-driven expansion of NK cells during viral infection, but not during bystander or homeostatic expansion.

IL-18 signaling is necessary for optimal IFN-γ production and maturation of NK cells during infection

Because IL-18 is required for optimal proliferation of NK cells during viral infection, we investigated additional NK cell effector functions that may be compromised in the absence of IL-18 signaling. At day 1.5 PI we observed that *Il18r1*$^{-/-}$ NK cells exhibited a defect in IFN-γ production, but not Granzyme B (Figure 2A), consistent with a previous report (31). Although both NK cell populations became similarly activated at days 1.5 and 7 PI (Supplementary Figure 3.1C and 1D), WT NK cells were found to be more mature, as measured by CD27 and CD11b staining, compared to *Il18r1*$^{-/-}$ cells (Figure 3.2B), suggesting IL-18 may have a role in promoting the maturation of activated NK cells.

Adoptive co-transfer and bone marrow chimeric studies revealed an expansion defect in the *Il18r1*$^{-/-}$ NK cell response to MCMV infection, which could be a consequence of decreased proliferation or increased apoptosis of *Il18r1*$^{-/-}$ NK cells relative to WT NK cells. We were not able to detect differences in BrdU incorporation, Ki67 staining, or CFSE dilution by WT and *Il18r1*$^{-/-}$ NK cells in MCMV-infected bone marrow chimeric mice (data not shown), consistent with a prior study (94). In addition, FLICA incorporation studies did not reveal any differences in pan-caspase activity between *Il18r1*$^{-/-}$ and WT NK cells on day 7 PI

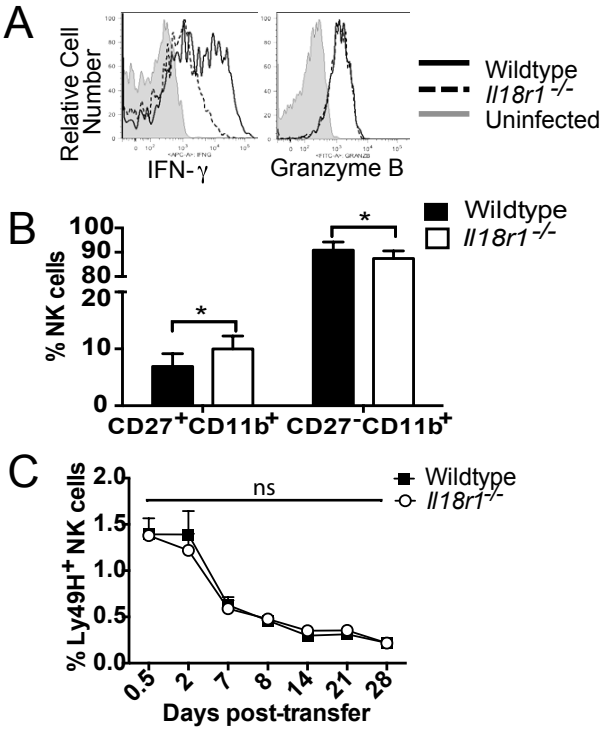


Figure 3.2. IL-18 is necessary for optimal NK cell maturation and IFN-γ production following MCMV infection. A. Mixed WT:*Il18r1*$^{-/-}$ chimeric mice were infected with MCMV and amount of IFN-γ and granzyme B in NK cells at day 1.5 PI are shown. **B.** WT and *Il18r1*$^{-/-}$ NK cells were co-transferred into *Ly49h*$^{-/-}$ mice and infected with MCMV. CD27 and CD11b staining is shown for WT and *Il18r1*$^{-/-}$ Ly49H^{+} NK cells at day 7 PI. **C.** Equal numbers of purified effector WT and *Il18r1*$^{-/-}$ NK cells (at day 7 PI) were co-transferred into a naïve *Ly49h*$^{-/-}$ host. Percentages of Ly49H^{+} NK cells are shown. Data are mean ± s.e.m. representative of at least 3 independent experiments with at least n=3 biological replicates per condition. * $p <$ 0.05 and ns, not significant, paired Student *t*-test.

(Supplementary Figure 3.1E), suggesting that *Il18r1*$^{-/-}$ NK cells were not more susceptible to apoptosis. Lastly, when WT and *Il18r1*$^{-/-}$ effector NK cells were sorted from MCMV-infected hosts on day 7 PI, and equal numbers co-transferred into naïve recipients, the two populations contracted at similar rates (Figure 3.2C), indicating that *Il18r1*$^{-/-}$ NK cells are not undergoing increased cell death at later time points, and that IL-18 signaling does not regulate the contraction phase or maintenance of memory NK cells.

Antiviral NK cell response depends on MyD88, but not the IL-1 receptor

Because the IL-18 receptor requires the adapter molecule MyD88 for downstream signaling (95), we investigated the contribution of MyD88 to the NK cell response to MCMV infection. WT:*Myd88*$^{-/-}$ mixed bone marrow chimeric mice were generated and both NK cell populations reconstituted similarly (data not shown). Following MCMV infection, *Myd88*$^{-/-}$ Ly49H^{+} NK cells exhibited a cell-intrinsic expansion defect compared to WT NK cells (Figure 3.3A), similar to *Il18r1*$^{-/-}$ NK cells (Figure 1C). In addition, fewer *Myd88*$^{-/-}$ NK cells produced IFN-γ after MCMV infection despite comparable upregulation of CD69 (Figure 3.3B). Similar to *Il18r1*$^{-/-}$ NK cells, *Myd88*$^{-/-}$ NK cells failed to mature as efficiently as WT NK cells at day 7 PI (Figure 3.3C). When equal numbers of WT and *Myd88*$^{-/-}$ NK cells were transferred into *Ly49h*$^{-/-}$ hosts followed by infection with MCMV, we observed preferential expansion and memory cell formation of the WT NK cells compared to *Myd88*$^{-/-}$ NK cells (Figure 3.3D). The IL-1 receptor, which is expressed by NK cells, is also known to use MyD88 for signaling (95); however, unlike *Il18r1*$^{-/-}$ or *Myd88*$^{-/-}$

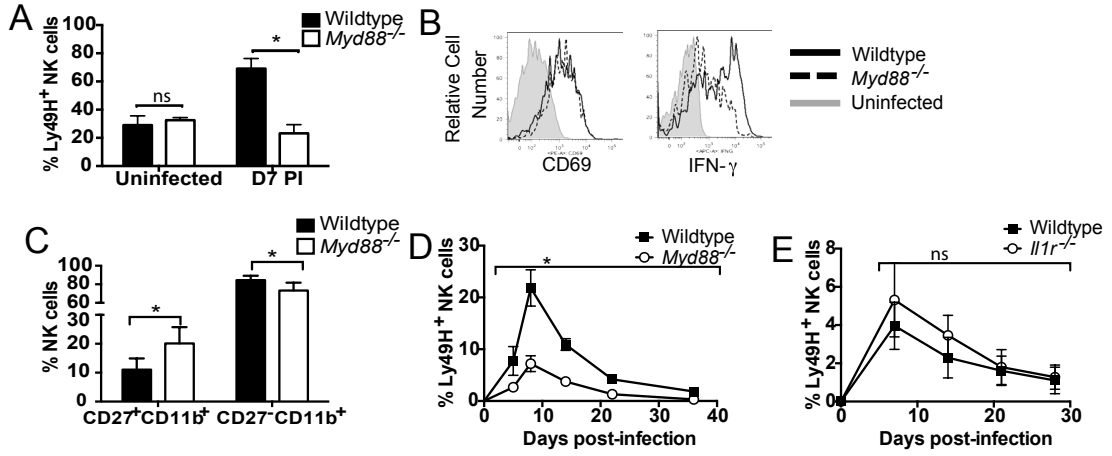


Figure 3.3. MyD88-deficient NK cells exhibit defective proliferation during MCMV infection. A. WT: *Myd88*$^{-/-}$ chimeric mice were infected with MCMV and percentages of splenic NK cells are shown for uninfected and day 7 PI. **B.** CD69 and IFN-γ are shown for wildtype and *Myd88*$^{-/-}$ NK cells (compared to uninfected mice) at day 1.5 PI. **C.** CD27 and CD11b staining is shown for WT and *Il18r1*$^{-/-}$ Ly49H^{+} NK cells at day 7 PI. **D.** Percentages of co-transferred WT (CD45.1) and *MyD88*$^{-/-}$ (CD45.2) Ly49H^{+} NK cells are shown following MCMV infection. **E.** Percentages of co-transferred WT and *Il1r*$^{-/-}$ Ly49H^{+} NK cells are shown during MCMV infection. Data are mean ± s.e.m. representative of three independent experiments with at least n=3 biological replicates per condition. * $p < 0.05$ and ns, not significant, paired Student *t*-test.

NK cells, *Il1r*$^{-/-}$ NK cells expanded comparably to WT NK cells at day 7 PI (Figure 3.3E) and showed similar effector function and phenotype as WT NK cells at days 1.5 and 7 PI (Supplementary Figures 3.2A-C). These findings support a mechanism for IL-18 signaling on NK cells through a MyD88-dependent signaling axis.

IL-12 signaling and STAT4 increase expression of MyD88 in activated NK cells

During MCMV infection, we observed an increase in *Myd88* expression in sorted NK cells by microarray and qRT-PCR (Figure 3.4A, data not shown). Given the important early role of IL-12 in activating NK cells, and a report that proinflammatory cytokines induce IL-18 signaling components in human NK cells (96), we investigated whether IL-12 directly influences the expression of the IL-18 signaling machinery. Overnight stimulation of sorted NK cells with IL-12 and IL-18 also showed an increase in the expression of *Myd88* by qRT-PCR (Figure 3.4B). STAT4 is a transcription factor that mediates signals downstream from the IL-12 receptor (97), and analysis of the *Myd88* promoter revealed one putative STAT4 binding site upstream of the transcriptional start site (Figure 3.4C). STAT4 ChIP followed by qRT-PCR identified a significant enrichment of STAT4 binding directly upstream of the *Myd88* transcriptional start site (Figure 3.4D). This increase in STAT4 binding was dependent on IL-12 stimulation, suggesting *Myd88* is a target gene of IL-12 signaling. Finally, we compared the expression of *Myd88* in WT and *Stat4*$^{-/-}$ NK cells during MCMV infection. Activated *Stat4*$^{-/-}$ NK cells exhibited lower levels of *Myd88* expression compared to WT NK cells. Altogether, these data indicate that IL-12 signaling acts through STAT4 to increase the

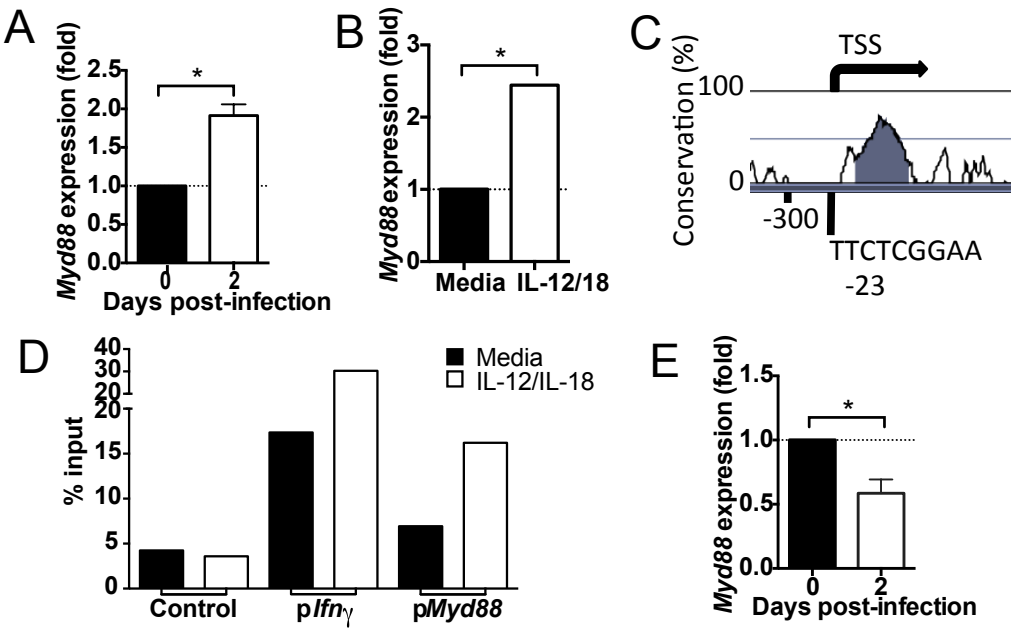


Figure 3.4. IL-12 signaling induces expression of *Myd88* in NK cells. A. qRT-PCR analysis of *Myd88* mRNA abundance in NK cells sorted from the spleen of WT mice following infection with MCMV (n=4 biological replicates per condition). Fold expression is shown relative to naïve mice. **B**. qRT-PCR analysis of *Myd88* mRNA abundance in WT NK cells stimulated with IL-12 and IL-18 for 18 h (n=3 biological replicates per condition). Fold expression is shown relative to medium only. **C**. Vista browser image of mouse *Myd88* promoter showing predicted STAT4 binding site. **D**. STAT4 binding at *Myd88*, *Ifng*, and control promoters as assessed through ChIP followed by qPCR in sorted WT NK cells stimulated with IL-12 and IL-18 for 18 h. STAT4 occupancy as percent of input is shown for target (*Myd88*) and control DNA (negative control: average of gene desert 50 kb upstream of *Foxp3*, *Zfp42*, and *Utf1* promoters; positive control: *Ifng* promoter). Data were confirmed in two independent experiments. E. qRT-PCR analysis of *Myd88* mRNA abundance in *Stat4*$^{-/-}$ NK cells relative to WT NK cells from mice infected with MCMV (n=4 biological replicates per condition). Data are mean ± s.e.m. representative of three independent experiments. * $p < 0.05$, paired Student *t*-test.

expression of *Myd88*, identifying a novel role for IL-12 in potentiating IL-18 signaling early during MCMV infection.

IL-18 signaling is dispensable for recall responses by memory NK cells

To assess whether IL-18 signaling regulates the functional capabilities of memory NK cells, we stimulated WT and *Il18r1*$^{-/-}$ memory NK cells with Ly49D, Ly49H, or IL-12 and IL-18 and measured their ability to degranulate and to make IFN-γ. Similar to resting NK cells, *Il18r1*$^{-/-}$ memory NK cells produced less IFN-γ than their WT counterparts only when stimulated with pro-inflammatory cytokines, but not with Ly49D, Ly49H or PMA and ionomycin (Figure 3.5A and data not shown). *Il18r1*$^{-/-}$ memory NK cells degranulated similarly to WT (Figure 3.5B). Thus, like resting NK cells (48), memory NK cells continue to depend on IL-18 for IL-12-mediated IFN-γ production.

Given the importance of IL-18 signaling on the optimal expansion of naïve NK cells during MCMV infection, we investigated its role during a recall response. Equal numbers of effector WT and *Il18r1*$^{-/-}$ NK cells were co-transferred and "parked" in a naïve host for three weeks, followed by infection with MCMV. Surprisingly, *Il18r1*$^{-/-}$ memory NK cells expanded similarly to their WT counterparts during secondary challenge by MCMV (Figure 3.5C), demonstrating a stage-specific requirement for IL-18 signals for proliferation. Following the peak of expansion, both cell populations contributed to equal frequencies of secondary memory NK cells. Thus, IL-18 signaling is specifically required for the primary

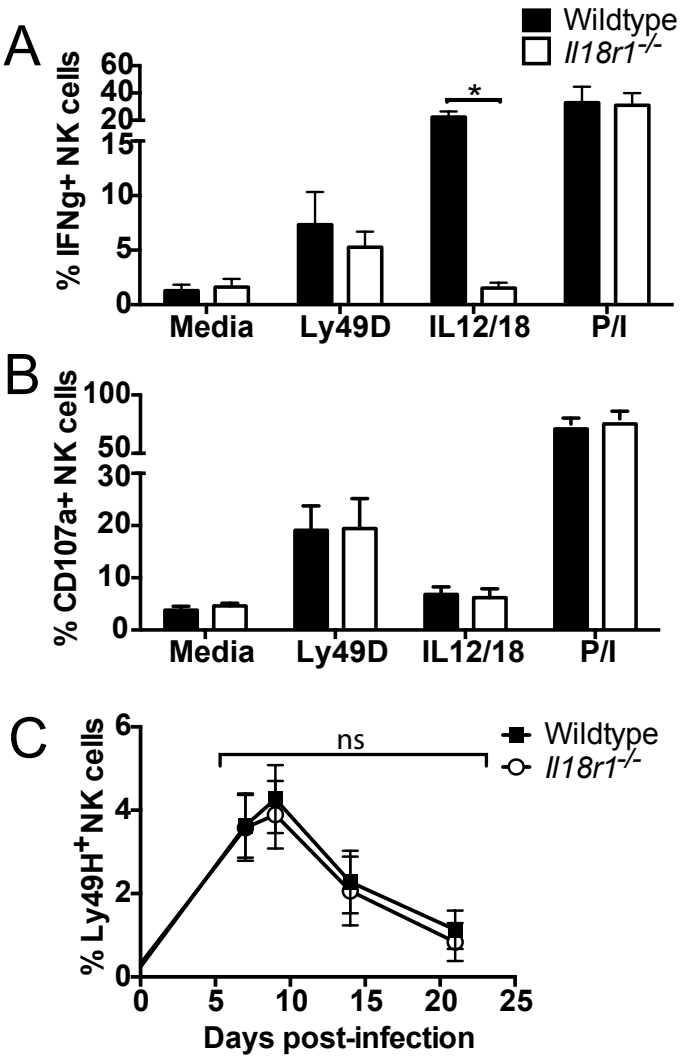


Figure 3.5. IL-18 signaling is dispensable for recall response of NK cells. Memory WT and *Il18r1*$^{-/-}$ NK cells were stimulated with Ly49D, IL-12 and IL18, or PMA and Ionomycin, and percent IFN-γ (**A**) or CD107a (**B**) expression shown. **C.** Percentage of recalled WT and *Il18r1*$^{-/-}$ NK cells (at day 28 PI) are shown following secondary challenge with MCMV. Data are mean ± s.e.m. representative of five independent experiments with at least n=4 biological replicates per condition. * $p < 0.05$ or ns, not significant, paired Student *t*-test.

expansion of virus-specific NK cells, but is dispensable in the subsequent recall response.

NK cells possess adaptive immune qualities in response to cytokine treatment (25) or exposure to pathogen and non-pathogen antigens (65). It is poorly understood what the cytokine signal requirements are in resting and memory NK cells. Here, we find a stage-specific requirement for IL-18, where IL-18 promotes the antigen-specific primary expansion of resting NK cells, but not the recall response of memory NK cells. Furthermore, we have identified a previously unknown role for IL-12 in promoting the expression of *Myd88* to enhance the IL-18 signaling cascade, and possibly sensitize NK cells to lower levels of IL-18 cytokine.

Our current findings suggest that as naïve NK cells differentiate into antigen-experienced memory cells, they become "specialized" and may rely more on antigen-specific signals and less on pro-inflammatory cytokine signals for clonal-like expansion. This mechanism would allow previously antigen experienced NK cells to respond more robustly during subsequent pathogen encounter. Because memory and recall NK cell responses have also been documented in humans following viral infection (89), determining whether a homologous role for IL-18 in the recall response of human NK cells may impact the use of this cytokine in clinical settings.

Materials and methods

Mice and infections

All mice used in this study were bred and maintained at MSKCC in accordance with IACUC guidelines. Mixed bone marrow chimeric mice were generated, and adoptive transfer studies and viral infections were performed as previously described (13).

Flow cytometry and cell sorting

Fc receptors were blocked with 2.4G2 mAb before staining with the indicated surface or intracellular antibodies (BD, BioLegend, or eBioscience). Flow cytometry was performed on an LSR II (BD). Cell sorting was performed on an Aria II cytometer (BD). All data were analyzed with FlowJo software (TreeStar). NK cell enrichment and adoptive transfers were performed as previously described (91).

qRT-PCR and ChIP

qRT-PCR and chromatin immunoprecipitation (ChIP) were performed as previously described (92). The following qRT- PCR primers were used: Myd88, *For: 5'-CACCTGTGTCTGGTCCATT-3', Rev: 5'-AGGCTGAGTGCAAACTTG-3';* Actb, *For: 5'-TGCGTGACATCAAAGAGAAG-3', Rev: 5'-CGGATGTCAACGTCACACTT-3'. The following qPCR primers were used for ChIP studies:* Myd88 promoter, *For: 5'-AAGTAGGAAACTCCACAGGCGAGC-3',*

Rev: 5'-TTCAAGAACAGCGATAGGCGGC-3'; Gene desert 50 kB upstream of Foxp3*, For: 5'-TAGCCAGAAGCTGGAAAGAAGCCA-3', Rev: 5'-TGATACCCTCCAGGTCCAACCATT-3';* Zpf42 promoter*, For: 5'-AGAGGGCGGTGTGTACTGTGGTG-3', Rev: 5'-CTTCTTCTTGCACCCGGCTTGAG-3';* Utf1 promoter, *For: 5'-AGTCGTTGAATACCGCGTTGCTG-3', Rev: 5'-CTGTTGAGATGTCGCCCAAGTGC-3';* Ifng promoter, *For: 5'-GCTCTGTGGATGAGAAAT-3', Rev: 5'-GCTCTGTGGATGAGAAAT-3'.*

Ex vivo stimulation of NK cells

Purified NK cells were stimulated for 4 h (memory cells) or 18 h (for ChIP), as previously described (92). Negative and positive controls include NK cells incubated with media only, or with PMA (50 ng/mL) and Ionomycin (1 μg/mL), respectively.

Statistical methods

All graphs depict mean ± s.e.m. Two-tailed paired Student's *t*-test was used to derive statistical differences. A p value < 0.05 was considered significant. Plots and statistical analyses were produced in GraphPad Prism.

CHAPTER 4

TYPE I IFN PROMOTES NK CELL EXPANSION DURING VIRAL INFECTION BY PROTECTING NK CELLS AGAINST FRATRICIDE

Introduction

Type I IFNs provide a potent line of anti-viral defense through direct and indirect effects on cells of the immune system leading to their activation and effector function (55, 98), and resulting in the attenuation of viral replication (99). IFN-α and IFN-β are among the most-studied members of the type I IFN family. All members of the type I IFN family signal through a ubiquitously expressed heterodimeric receptor that is composed of the IFNAR1 and IFNAR2 chains. Type I IFNs act directly on NK cells to promote their activation, cell-cycle entry, and cytotoxic function during viral infection (40, 54-56). However, the experimental systems employed in these previous studies – direct infection of cytokine receptor-deficient mice or wildtype mice with cytokine neutralization – are complicated by potential differences in degree of inflammation, indirect cytokine effects, and viral load. Thus, efforts to delineate the direct influence of type I IFN on anti-viral NK cell responses, while eliminating pleiotropic effects, are lacking.

Although substantial amounts of type I IFN are produced during viral infection, this cytokine is constitutively present at basal levels and affects the development and homeostasis of various hematopoietic lineages (100-102). An indirect effect of type I IFN on NK cell development and maturation has been described recently (103, 104). Because the prolific expansion and generation of memory NK cells during MCMV infection are dependent predominantly on the pro-

inflammatory cytokines IL-12 and IL-18 (91, 105, 106), it was of interest whether type I IFNs play a role in these processes. Here, we use mice deficient in the IFNAR1 chain (*Ifnar*$^{-/-}$) in an adoptive co-transfer system and in bone marrow chimeric mice to investigate the direct influence of type I IFN signaling on NK cells responding against MCMV infection.

Results and Discussion

Type I IFN and STAT1 are required for optimal NK cell responses following MCMV infection

Given the pleiotropic effects of type I IFNs (107), we assessed the ability of *Ifnar*$^{-/-}$ and wildtype (WT) Ly49H^{+} NK cells to expand in response to MCMV infection using an adoptive co-transfer system (91), where both transferred NK cell populations respond against virus and experience similar inflammatory cues within the same host environment. WT and *Ifnar*$^{-/-}$ NK cells were co-transferred into Ly49H-deficient mice, whose NK cells are unable to recognize the virus-encoded glycoprotein m157 during MCMV infection and undergo clonal expansion (13). In contrast to the WT NK cells that robustly expanded following MCMV infection, *Ifnar*$^{-/-}$ NK cells failed to expand robustly (Figure 4.1A). Although they exhibited an expansion defect, *Ifnar*$^{-/-}$ NK cells were able to mature nearly as well as WT NK cells in response to MCMV infection, as evidenced by the downregulation of CD27 and upregulation of CD11b and KLRG1 (Figures 4.1B and C). We also investigated

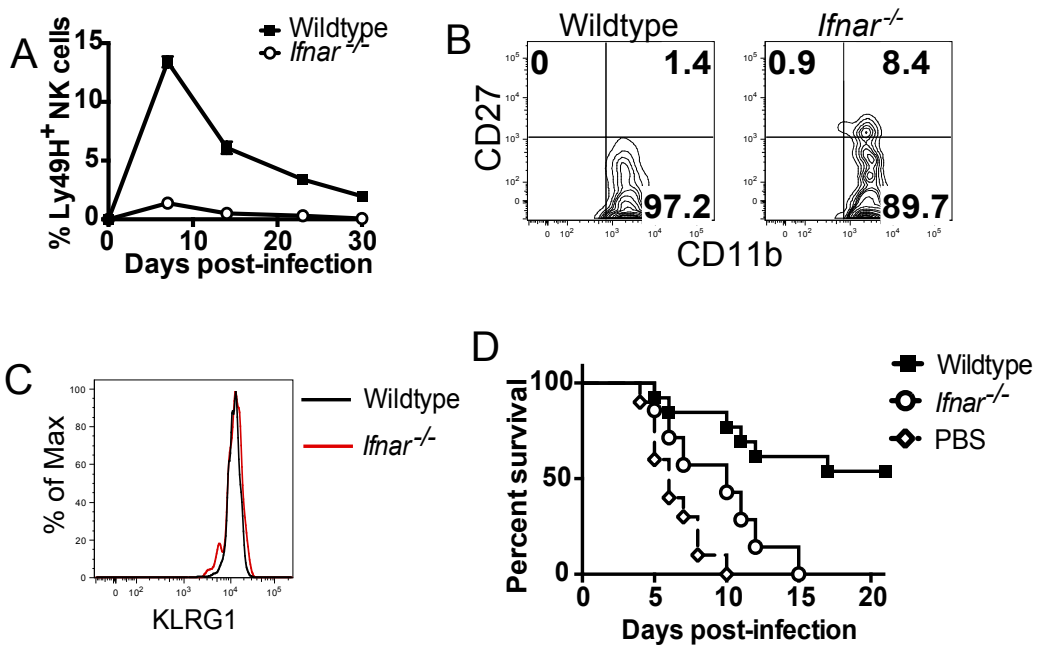


Figure 4.1. Type I IFN is essential for a robust and protective anti-viral NK cell response following MCMV infection. (**A**) WT and *Ifnar*$^{-/-}$ NK cells were co-transferred into a Ly49H-deficient host and infected with MCMV. Percentages of Ly49H^{+} NK cells are shown. (**B-C**) CD27 versus CD11b, and KLRG1 staining are shown for WT and *Ifnar*$^{-/-}$ Ly49H^{+} NK cells at day 7 PI. (**D**) Neonatal mice received 10^{6} WT (n=13) or *Ifnar*$^{-/-}$ (n=7) NK cells followed by MCMV infection. Control mice received PBS (n=10). The percentage of surviving mice is shown for each group. Data were pooled from three experiments, and represent mean ± s.e.m. of at least three independent experiments with at least n=3 biological replicates per condition.

the contribution of type I IFN signaling in NK cells for protection against lethal MCMV challenge. Equal numbers of naïve WT or *Ifnar*$^{-/-}$ NK cells were transferred into separate neonatal mice and then challenged with MCMV. In contrast to mice receiving WT NK cells, which protected approximately 50% of recipients, all mice receiving *Ifnar*$^{-/-}$ NK cells succumbed to infection by day 15 post-infection (PI) (Figure 4.1D), highlighting the importance of type I IFN signaling specifically in NK cells for protective immunity against viral challenge.

Type I IFNs signal through STAT1-STAT2 heterodimers and STAT1-STAT1 homodimers (108). Therefore, we determined the role of STAT1 in the NK cell response to MCMV infection using STAT1-deficient mice. Equal numbers of WT and *Stat1*$^{-/-}$ Ly49H^{+} NK cells were co-transferred into Ly49H-deficient hosts and then infected with MCMV. Similar to *Ifnar*$^{-/-}$ NK cells, *Stat1*$^{-/-}$ NK cells exhibited a striking defect in expansion during the immune response to MCMV infection (Figure 4.2A), even though *Stat1*$^{-/-}$ NK cells were able to mature nearly as well as WT NK cells following infection (Figures 4.2B and C). These data support the importance of STAT1-mediated type I IFN signaling in NK cells for an optimal antiviral NK cell response.

Type I IFN is required for activation and effector function in NK cells following MCMV infection

Type I IFN has both direct and indirect effects on NK cell development and maturation (103, 104). In accordance with these prior reports, resting splenic NK cells from *Ifnar*$^{-/-}$ mice exhibited an increase in immature NK cells as indicated by

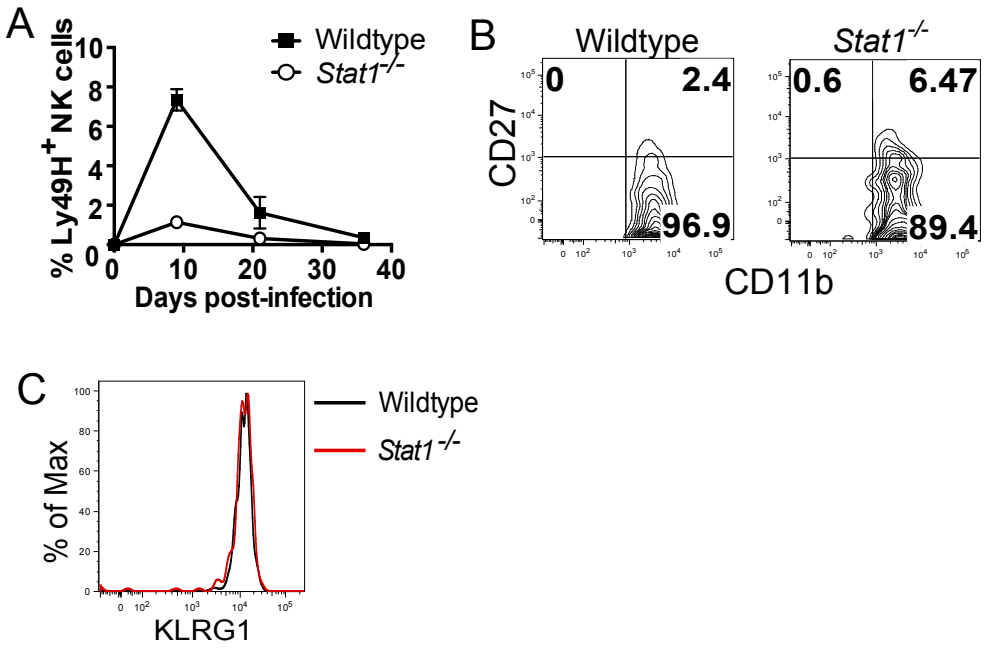


Figure 4.2. *Stat1*$^{-/-}$ NK cells have a defective expansion during MCMV infection. (**A**) WT and *Stat1*$^{-/-}$ NK cells were co-transferred into a Ly49H-deficient host and infected with MCMV. Percentages of Ly49H NK cells are shown. **(B-C)** CD27 versus CD11b, and KLRG1 staining are shown for WT and *Stat1*$^{-/-}$ Ly49H^{+} NK cells at day 7 PI. Data are mean ± s.e.m. and representative of at least 3 independent experiments with at least n=3 biological replicates per condition.

CD27, CD11b, and KLRG1 expression when compared to WT (data not shown). Thus, we generated mixed WT:*Ifnar*$^{-/-}$ bone marrow chimeras, where development of *Ifnar*$^{-/-}$ NK cells appeared grossly normal (data not shown), and investigated the role of type I IFNs on the NK cell response against MCMV infection. At day 1.5 following MCMV infection, *Ifnar*$^{-/-}$ NK cells in mixed chimeric mice exhibited defective upregulation of CD69 (Figure 4.3A), a marker of activation downstream of type I IFN signaling (109). *Ifnar*$^{-/-}$ NK cells also failed to upregulate granzyme B compared to their WT counterparts at day 1.5 PI (Figure 4.3B), consistent with a previous study that found a requirement for type I IFN signaling in NK cells for the induction of cytotoxicity following MCMV infection (40). Interestingly, *Ifnar*$^{-/-}$ NK cells produced more IFN-γ than WT NK cells at day 1.5 PI (Figure 4.3B). STAT1 phosphorylation was completely ablated in NK cells that cannot sense type I IFNs (Figure 4.3C). These findings demonstrate the ability of type I IFNs to directly impact the expression of key effector molecules in NK cells, likely via robust STAT1 phosphorylation.

Given the rescue of the maturation defect of *Ifnar*$^{-/-}$ NK cells in mixed bone marrow chimeric mice (104), we assessed the ability of Ly49H^{+} *Ifnar*$^{-/-}$ and *Stat1*$^{-/-}$ NK cells from mixed chimeras to undergo clonal expansion in response to MCMV infection. Following adoptive transfer and infection, *Ifnar*$^{-/-}$ NK cells were unable to expand as robustly as their WT counterparts (Figure 4.3D). Similarly, mixed chimera-derived *Stat1*$^{-/-}$ NK cells also exhibited a marked defect in expansion following MCMV infection compared to WT NK cells (Figure 4.3E). Long-lived NK were also diminished in NK cell populations that lack IFNAR or STAT1

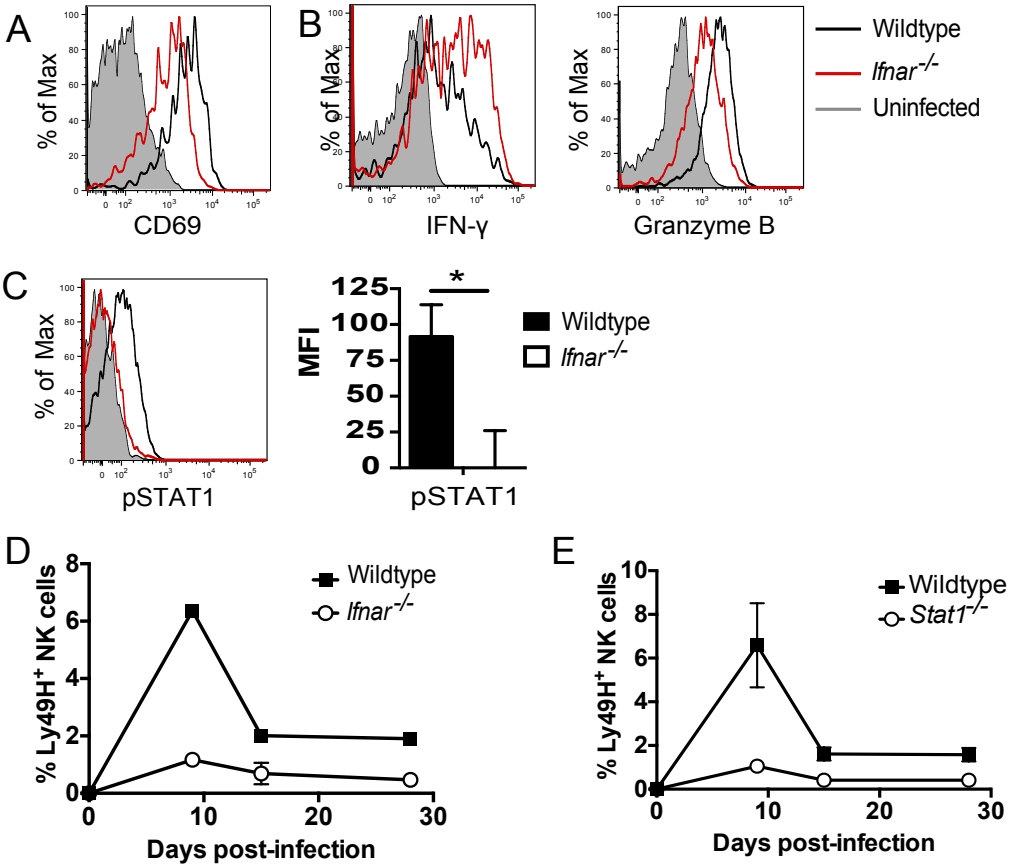


Figure 4.3. Type I IFN promotes NK cell activation and expression of effector molecules following MCMV infection. (**A-B**) WT: *Ifnar*$^{-/-}$ chimeric mice were infected with MCMV. CD69, IFN-γ, and granzyme B are shown for splenic wildtype and *Ifnar*$^{-/-}$ NK cells (compared to uninfected mice) at day 1.5 PI. (**C**) STAT1 phosphorylation of NK cells at day 1.5 PI is shown, and bar graphs plot MFI. (**D-E**) Ly49H^{+} NK cells from WT: *Ifnar*$^{-/-}$ chimeric mice (**D**) or WT:*Stat1*$^{-/-}$ chimeric mice (**E**) were transferred into Ly49H-deficient hosts and infected with MCMV. Percentages of Ly49H^{+} NK cells are shown. Data are mean ± s.e.m. and representative of three independent experiments with at least n=3 biological replicates per condition. * p <0.05 and ns, not significant, paired Student t-test.

(Figure 4.3D-E), highlighting the critical role of type I IFN signaling in NK cells for a robust viral-specific response.

Type I IFN is dispensable for NK cell proliferation, but shields NK cells against apoptosis following MCMV infection

We explored two possibilities for the expansion defect of *Ifnar*$^{-/-}$ NK cells in response to MCMV infection: proliferation and apoptosis. To investigate whether a proliferative defect exists in NK cells that are unable to sense type I IFNs, Cell Trace Violet (CTV)-labeled Ly49H^{+} NK cells from WT:*Ifnar*$^{-/-}$ chimeras were transferred into Ly49H-deficient hosts and infected with MCMV. *Ifnar*$^{-/-}$ NK cells exhibited a modest increase in proliferation when compared to WT NK cells at day 4 PI (Figure 4.4A), suggesting type I IFN signals actually restrain NK cell proliferation following activation. We next compared the amount of apoptosis in *Ifnar*$^{-/-}$ and WT NK cells that are activated during MCMV infection. Following adoptive transfer and MCMV infection, *Ifnar*$^{-/-}$ NK cells incorporated significantly more FLICA, a measurement of activated caspases, than WT NK cells at day 4 PI (Figure 4.4B, 2.1% ± 0.5 vs. 12.0 ± 1.8, respectively; p=0.006). Together, these findings suggest that type I IFNs shield activated NK cells from cell death during viral infection.

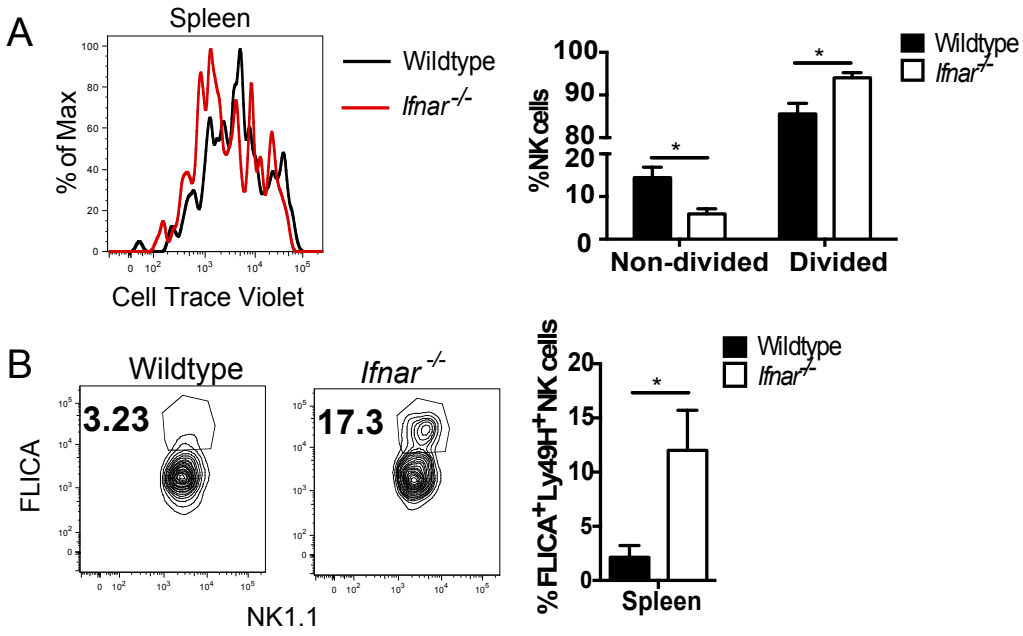


Figure 4.4. *Ifnar*$^{-/-}$ NK cells proliferate normally but undergo greater apoptosis during MCMV infection. (**A**) Ly49H^{+} NK cells from WT: *Ifnar*$^{-/-}$ chimeric mice were labeled with 5 μM CTV, transferred into a Ly49H-deficient host, and infected with MCMV. Proliferating cells were analyzed at day 4 PI. Bar graph shows percentages of divided and non-divided NK cells at day 4 PI for each group. (**B**) FLICA incorporation is shown in plots, and bar graph shows percentages of FLICA^{+} NK cells in each group. Data are mean ± s.e.m., and representative of three independent experiments with at least n=3 biological replicates per condition. * $p <$ 0.05 and ns, not significant, paired Student *t*-test.

Type I IFN protects NK cells from fratricide via a perforin- and NKG2D-dependent mechanism

To evaluate whether host NK cells were killing *Ifnar*$^{-/-}$ NK cells, we transferred equal numbers of WT and *Ifnar*$^{-/-}$ NK cells into WT or perforin-deficient (*Prf1*$^{-/-}$) hosts and infected with lymphocytic choriomeningitis virus (LCMV). LCMV infection elicits a strong type I IFN response (90), but does not drive the antigen-specific proliferation of Ly49H^{+} NK cells observed during MCMV infection, thus ruling out the influence of the m157-Ly49H interaction in determining relative NK cell numbers. Following adoptive transfer and LCMV infection in WT hosts, WT NK cells persisted whereas the percentage of *Ifnar*$^{-/-}$ NK cells diminished over time (Figure 4.5A). However, *Ifnar*$^{-/-}$ NK cells transferred into *Prf1*$^{-/-}$ hosts were able to persist, and interestingly larger percentages were observed compared to the co-transferred WT NK cell population (Figure 4.5A), uncovering a novel role for type I IFNs in protecting NK cells against perforin-mediated elimination. To confirm that the elimination of *Ifnar*$^{-/-}$ NK cells in WT mice was due to host NK cells, and not CD8^{+} T cells or another cell source, we transferred equal numbers of WT and *Ifnar*$^{-/-}$ NK cells into WT or *NKp46*^{Cre} x R26^{DTA} hosts and infected with LCMV. *NKp46*^{Cre} x R26^{DTA} mice express the Cre recombinase under the control of the *NKp46* promoter and possess a *loxP*-flanked stop cassette followed by a diphtheria toxin A, thus creating a host where all NK cells are ablated. LCMV infection revealed a predominance of transferred WT NK cells compared to *Ifnar*$^{-/-}$ NK cells in WT hosts, which was not observed in *NKp46*^{Cre} x R26^{DTA} hosts (Figure 4.5B). Similar to the perforin-deficient hosts, *NKp46*^{Cre} x R26^{DTA} hosts revealed a higher

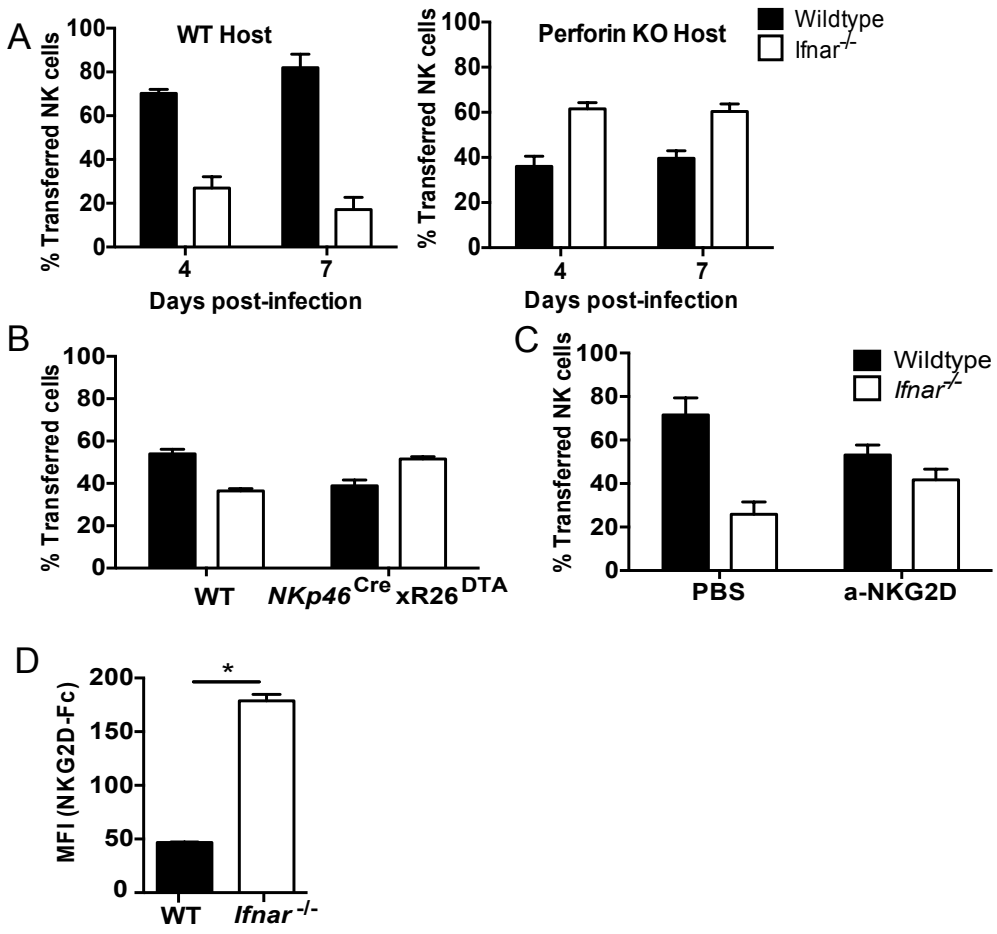


Figure 4.5. Type I IFN protects against perforin- and NKG2D-dependent NK cell fratricide during viral infection. (**A**) NK cells from WT:*Ifnar*$^{-/-}$ chimeric mice were labeled with CTV and transferred into WT or *Prf1*$^{-/-}$ hosts followed by infection with LCMV. Bar graph shows percentages of transferred cells for each group at different points during infection in blood. (**B**) NK cells from WT:*Ifnar*$^{-/-}$ chimeric mice were labeled with CTV and transferred into WT or *NKp46*^{Cre} x R26^{DTA} hosts, followed by LCMV infection. Bar graph shows percentages of transferred cells for each group in spleen at day 3 PI. (**C**) NK cells from WT:*Ifnar*$^{-/-}$ chimeric mice were labeled with CTV and transferred into WT hosts receiving anti-NKG2D antibody or PBS, followed by LCMV infection. Bar graph shows percentages of transfer cells for each group in spleen at day 3 PI. (**D**) WT:*Ifnar*$^{-/-}$ chimeric mice were directly infected with LCMV. Expression of NKG2D ligands is shown at day 2 PI. Data are mean ± s.e.m. and representative of 3 independent experiments with at least n=3 biological replicates per condition. * p <0.05 or ns, not significant, paired Student t-test.

percentage of *Ifnar*$^{-/-}$ NK cells compared to WT NK cells following infection. Thus, these findings support a perforin-dependent NK cell-mediated elimination of NK cells that are unable to sense type I IFNs during viral infection.

In order to further uncover the mechanism behind NK cell-mediated fratricide, we investigated NKG2D triggering in NK cells as a potential activating signal involved in the killing of *Ifnar*$^{-/-}$ NK cells during viral infection. Equal numbers of WT and *Ifnar*$^{-/-}$ NK cells were co-transferred into WT hosts that received a non-depleting, neutralizing anti-NKG2D-blocking antibody (or PBS as a negative control) and then infected with LCMV. As expected, WT NK cells preferentially survived compared to *Ifnar*$^{-/-}$ NK cells in the PBS-treated control mice (Figure 4.5C). However, in mice receiving anti-NKG2D, similar persistence between WT and *Ifnar*$^{-/-}$ NK cells was observed (Figure 4.5C), suggesting that the NKG2D pathway may represent at least one mechanism that activates fratricide. Furthermore, we confirmed expression of NKG2D ligands on the NK cells soon after infection. Following LCMV infection of WT:*Ifnar*$^{-/-}$ bone marrow chimeras, *Ifnar*$^{-/-}$ NK cells expressed significantly higher amounts NKG2D ligand (Figure 4.5D). This finding suggests type I IFN may have a direct role in suppressing NKG2D ligand induction during viral infection. Taken together, these data indicate that type I IFN acts to combat NK cell-mediated fratricide that is dependent on NKG2D triggering and perforin release.

Type I IFN is dispensable for NK cell survival and memory formation during MCMV infection in mice lacking WT NK cells

If host NK cells are indeed mediating fratricide of transferred *Ifnar*$^{-/-}$ NK cells during viral infection, we hypothesized that *Ifnar*$^{-/-}$ NK cells should expand normally and generate long-lived memory following MCMV infection in a system devoid of NK cells. Equal numbers of Ly49H^{+} WT (CD45.1) and *Ifnar*$^{-/-}$ (CD45.2) NK cells from mixed chimeric mice were transferred into *Rag2*$^{-/-}$ *x Il2rg*$^{-/-}$ hosts (which lack T, B, and NK cells) and infected with MCMV. Following MCMV infection, *Ifnar*$^{-/-}$ NK cells expanded robustly, and even exhibited a moderate increase in percentage compared to WT NK cells at day 7 PI (Figure 4.6A). Activation and maturation of virus-specific *Ifnar*$^{-/-}$ NK cells was nearly identical to WT NK cells, as evidenced by CD27, CD11b, and KLRG1 expression (data not shown). Furthermore, *Ifnar*$^{-/-}$ NK cells could be recovered in comparable numbers and phenotype to WT NK cells at greater than 4 weeks PI (Figure 4.6B-D), revealing the ability of *Ifnar*$^{-/-}$ NK cells to generate a long-lived memory pool during infection in hosts lacking wildtype NK cells.

Traditionally classified as a member of the innate immune system, NK cells are known to provide a crucial line of early defense against viral infections in both humans and mice (110-112). In more recent years, NK cells have been described to possess many features of adaptive immunity (67, 113). However, the molecular mechanisms behind these adaptive responses are not well understood. Although type I IFN has long been known to mediate antiviral effects, partly through its

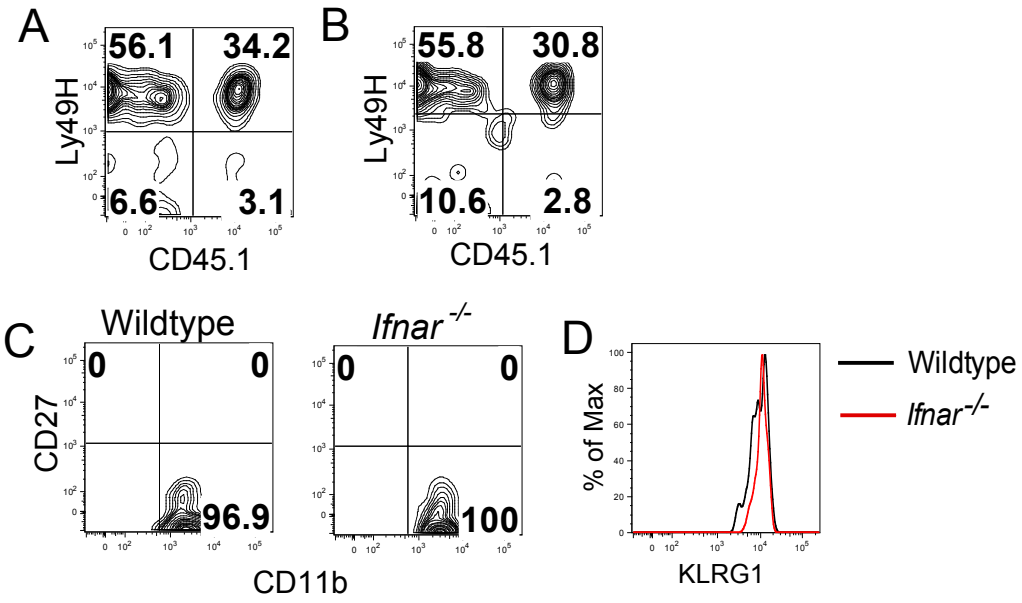


Figure 4.6. Type I IFN is dispensable for NK cell survival and memory formation in *Rag2 x Il2rg*$^{-/-}$ hosts. NK cells from WT:*Ifnar*$^{-/-}$ chimeric mice were co-transferred into *Rag2*$^{-/-}$ *x Il2rg*$^{-/-}$ hosts and infected with MCMV. Percentages and phenotype of WT (CD45.1) and *Ifnar*$^{-/-}$ (CD45.2) NK cells is shown at days 7 PI (**A**) and 30 PI (**B-E**).

activity on NK cells (40, 54), previous studies have failed to distinguish between the direct and indirect role of type I IFNs on NK cells during infection. Our current study demonstrates that type I IFN acts directly on NK cells to promote their survival by protecting them from elimination via NK cell-mediated fratricide.

We demonstrate that NK cells unable to sense type I IFNs can proliferate normally, but undergo more apoptosis than WT NK cells following MCMV infection. Although our findings suggests that a perforin- and NKG2D-dependent fratricide plays a major role in the increased cell death observed in *Ifnar*$^{-/-}$ NK cells during viral infection, additional survival mechanisms may be lacking in these cells. Type I IFN has been previously described to promote the survival of activated CD8^{+} T cells in a manner that is independent of Bcl-2 (114). Similarly, we found no difference in the expression of Bcl-2 between WT and *Ifnar*$^{-/-}$ NK cells following MCMV infection (data not shown). Thus, a Bcl-2-independent mechanism is promoting survival of antigen-specific NK cells that can sense type I IFNs, shielding these cells from apoptosis. Future studies investigating gene targets of STAT1 may reveal additional pro-survival mechanisms in NK cells exposed to type I IFNs during viral infection.

We previously demonstrated that the pro-inflammatory cytokine IL-12 and downstream signaling components STAT4 and Zbtb32 are critical for the clonal-like proliferation of Ly49H^{+} NK cells during MCMV infection (91, 92). Interestingly, the expansion defect in *Ifnar*$^{-/-}$ NK cells is nearly as pronounced as that of the *Il12rb*$^{-/-}$ NK cells, demonstrating a non-redundant role for these cytokines in promoting NK cell expansion. Based on our current findings, we propose a complementary role

for IL-12 and type I IFN in simultaneously driving cellular proliferation and protecting against cell death via fratricide, respectively, during virus-specific NK cell expansion. Given the use of type I IFN in the clinic and its potential impact in vaccination strategies, it is important to elucidate the direct and indirect roles this cytokine has on specific cellular compartments during the immune response. Our work brings us one step closer by uncovering a novel protective role of type I IFN signaling in NK cells during viral infection.

Materials and Methods

Mice and infections

All of the mice used in this study were bred and maintained at MSKCC in accordance with IACUC guidelines. Adoptive transfer studies, the generation of mixed bone marrow chimeric mice, and MCMV infections were performed as previously described (13). LCMV infection was performed as described (115). In vivo blockade of NKG2D signaling was accomplished by intraperitoneal injection of purified antibody for NKG2D (clone CX5, 200 μg/mouse) receptor at d0 PI LCMV infection. In proliferation assays, NK cells were labeled with 5 μM Cell Trace Violet (Invitrogen) before transfer.

Flow cytometry and cell sorting

Blocking of Fc receptors was performed with 2.4G2 mAb before staining with the indicated surface or intracellular antibodies (BD, BioLegend, or eBioscience). All

flow cytometry was performed on an LSR II (BD). The data were analyzed using FlowJo software (TreeStar). The enrichment and adoptive transfer of NK cells were performed as previously described (91).

Statistical methods

Mean ± s.e.m is depicted in all graphs. A two-tailed unpaired Student's t-test was used to determine statistical differences. Statistical significance was assigned to p values <0.05.All statistical analyses and plots were produced in GraphPad Prism.

CHAPTER 5

DISTINCT STAGE-SPECIFIC FUNCTION OF T-BOX TRANSCRIPTION FACTORS IN THE ANTIVIRAL NK CELL RESPONSE

Introduction

Although NK cells have been classically regarded as cells of innate immunity, they are now appreciated to possess developmental and functional traits in common with T and B cells of adaptive immunity (89). In particular, a subset of NK cells expressing the Ly49H receptor, which can bind the mouse cytomegalovirus (MCMV)-encoded glycoprotein m157, will undergo a clonal-like proliferation and generate long-lived memory cells (13) similar to antigen-specific $CD8^+$ T cells encountering pathogen-derived peptides presented on MHC class I. Recently, it has been demonstrated that pro-inflammatory interleukin (IL)-12 and STAT4 signals induce the pro-proliferative factor Zbtb32 (92), and all three are required for MCMV-specific expansion and "memory" of $Ly49H^+$ NK cells (91, 92). Further mechanisms governing the transcriptional regulation of NK cells during viral infection remains to be elucidated.

T-box family transcription factors Eomes and T-bet have wide-ranging effects that direct lymphocyte immunity. The role of T-bet and Eomes in the response of $CD8^+$ T cells against pathogens has been well characterized (58, 59). More recently, several groups have described the importance of Eomes and T-bet in NK cell development and function (58, 60-62). Similar to effector and memory $CD8^+$ T cells, mature NK cells exhibit constitutive T-bet and Eomes expression (60, 63). T-bet and Eomes have been described to control specific checkpoints of NK

cell development and maturation, with expression of DX5, loss of TRAIL, and gain of Ly49 receptor diversity (all characteristics of NK cell maturation) being dependent on Eomes (61). In the liver, T-bet was shown to control the development of a distinct Eomes$^-$ NK cell lineage (62). The role of these transcription factors on the antiviral response of mature NK cells has not been studied.

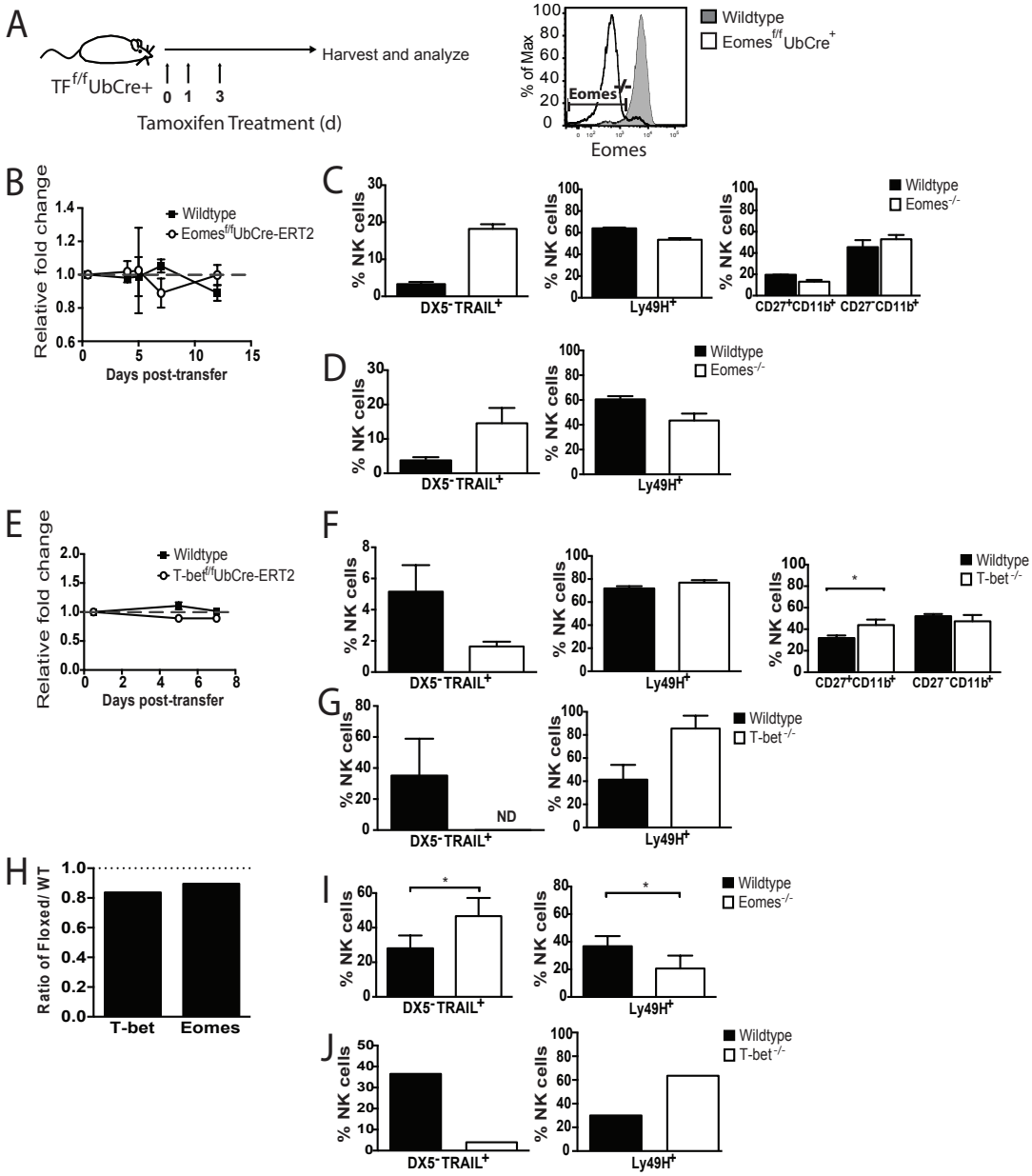
In this study, we use an inducible gene deletion system to probe the effects of single T-box family transcription factor ablation in Ly49H$^+$ NK cells at various time points during MCMV infection. We demonstrate that T-bet and Eomes possess non-redundant functions in the antiviral NK cell response and generation of NK cell "memory". Although both transcription factors were critical for the proliferative burst of virus-specific NK cells, only continuous T-bet expression was required during the memory maintenance phase for the persistence of virus-experienced NK cells.

Results

T-box transcription factors dispensable for homeostatic turnover and proliferation of NK cells

We generated mice containing a floxed Eomes or T-bet gene along with a Cre recombinase-human estrogen receptor ligand-binding domain (ERT2) fusion protein expressed downstream of the ubiquitin promoter (Ub) (*Eomes$^{f/f}$ x Ub$^{Cre\text{-}ERT2}$* or *Tbx21$^{f/f}$ x Ub$^{Cre\text{-}ERT2}$* mice, respectively). Treatment of *Eomes$^{f/f}$ x Ub$^{Cre\text{-}ERT2}$* or *Tbx21$^{f/f}$ x Ub$^{Cre\text{-}ERT2}$* mice with tamoxifen causes the specific excision of the floxed T-box transcription factor gene (Figure 5.1A and data not shown). This inducible

Figure 5.1. Eomes and T-bet are dispensable for NK cell homeostasis in both normal and lymphopenic mice. (**A**) Schematic of tamoxifen treatment. Mice were given a regimen of tamoxifen on days 0, 1, and 3. Control mice received oil. Panel shows expression of Eomes in NK cells three weeks after $Eomes^{f/f}$ $UbCre^{+}$ mice were given tamoxifen. Gates show $Eomes^{-/-}$ population in mice receiving tamoxifen. (**B-G**) WT (CD45.1) and $Eomes^{f/f}$ $UbCre^{+}$ (CD45.2, B-D) or $T\text{-}bet^{f/f}$ $UbCre^{+}$ (CD45.2, **E-G**) NK cells were co-transferred into WT (CD45.1x2) mice and treated with tamoxifen or oil at day 0 PT. The relative fold change of the floxed and WT NK cell ratio relative to their starting ratio is shown in panels B and E. Assessment of phenotypic markers at least one week post transfer for the spleen (**C** and **F**) and liver (**D** and **G**) are shown. Data are representative of two experiments with at least 3 mice in each condition. (**H**) WT (CD45.1) and $Eomes^{f/f}$ $UbCre^{+}$ (CD45.2) or $T\text{-}bet^{f/f}$ $UbCre^{+}$ (CD45.2) NK cells were co-transferred into *Rag*$^{-/-}$ *x Il2rg*$^{-/-}$ mice and treated with tamoxifen or oil. (**I** and **J**) The ratio of floxed and WT NK cells at day 9 PT relative to their starting ratio is shown. Assessment of phenotypic expression in the spleen is shown. Data are representative of one experiment, with 2-3 mice per condition. * $p < 0.05$ and ns, not significant, paired Student *t*-test.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CD27-CD11b+
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Ratio of Floxed/ WT
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gene deletion system allows us to individually delete Eomes or T-bet at steady state, and at different time points throughout MCMV infection, allowing us to distinguish the influence of individual T-box transcription factors on NK cell homeostasis and antiviral response. (Cells where Eomes or T-bet are ablated following tamoxifen treatment will be shown in figures as $Eomes^{-/-}$ or $T\text{-}bet^{-/-}$, respectively.)

We first investigated the role of Eomes expression in resting mature NK cells. We isolated resting NK cells from the spleen of $Eomes^{f/f}$ x $Ub^{Cre\text{-}ERT2}$ mice (CD45.2) and co-transferred them with equal numbers of WT NK cells (CD45.1) into CD45.1 x CD45.2 WT hosts. Host mice were immediately started on a tamoxifen regimen to induce Eomes deletion. Greater than one week following tamoxifen treatment, we found equal numbers of Eomes floxed and WT NK cells (Figure 5.1B), suggesting Eomes is not necessary for the maintenance of NK cell numbers under steady state conditions. However, NK cells that deleted Eomes exhibited an increase in the percentage $TRAIL^+$ cells and a decrease in the percentage $Ly49H^+$ cells in both the spleen and liver (Figure 5.1C-D). Using CD27 and CD11b as markers of NK cell maturation (116), no significant changes were observed between Eomes floxed and WT NK cells (Figure 5.1C). When $Tbx21^{f/f}$ x $Ub^{Cre\text{-}ERT2}$ NK cells were transferred with WT NK cells and hosts treated with tamoxifen, equal numbers of transferred populations were recovered one week later (Figure 5.1E), suggesting T-bet is also not essential for the maintenance of mature NK cells at steady state. In contrast to Eomes deletion, NK cells that deleted T-bet exhibited a decrease in the percentage of $TRAIL^+$ NK cells, and increase in the percentages of $Ly49H^+$ NK cells in the spleen and liver (Figure 5.1F-G). A

significant increase in the percentage of $CD27^+$ $CD11b^+$ cells was also observed (Figure 5.1F). In all experiments, a control set of mice receiving oil only (without tamoxifen) showed no phenotypic differences between floxed and WT cells (data not shown). Similar observations were made when resting splenic NK cells from floxed and WT mice were transferred into lymphopenic *Rag2*$^{-/-}$ *x Il2rg*$^{-/-}$ mice (where NK cells have been shown to undergo homeostatic proliferation (117) followed by tamoxifen treatment (Figure 5.1H-J). In accordance to a previous report (61), these data support the role of Eomes and T-bet in repressing and promoting TRAIL expression, respectively, and in the maintenance of a mature phenotype.

Eomes and T-bet are required for the expansion of virus-specific NK cells

To investigate the effects of Eomes and T-bet on the various stages of the virus-specific NK cell response we began by inducing the deletion of Eomes or T-bet directly before infection. We isolated resting $Ly49H^+$ NK cells from the spleen of *Eomes*$^{f/f}$ *x Ub*$^{Cre\text{-}ERT2}$ or *Tbx21*$^{f/f}$ *x Ub*$^{Cre\text{-}ERT2}$ mice (CD45.2) and co-transferred them with equal numbers of WT $Ly49H^+$ NK cells (CD45.1) into *Ly49h*$^{-/-}$ hosts that were immediately treated with tamoxifen (or oil as a control). Host mice were then infected with MCMV four days post-transfer (PT). We found Eomes to be critical for the expansion of $Ly49H^+$ NK cells during MCMV infection, with Eomes-deleted NK cells exhibiting a marked impairment in expansion compared to WT NK cells (Figure 5.2A-B). Current studies exploring the effect of Eomes expression on the phenotype of activated NK cells are underway.

Similar to Eomes deletion, when T-bet was deleted in NK cells from *Tbx21*$^{f/f}$ *x Ub*$^{Cre\text{-}ERT2}$ mice, transferred effector and memory $Ly49H^+$ NK cell numbers were

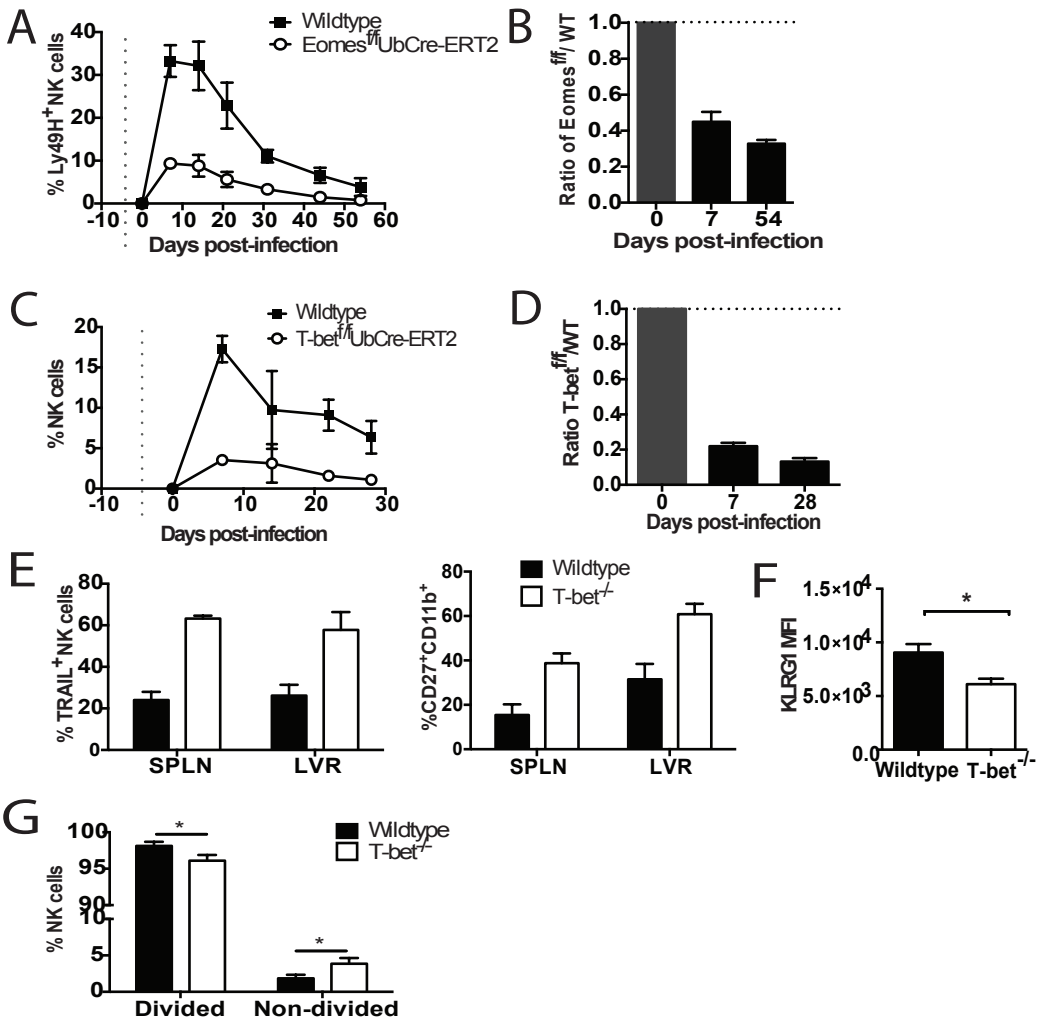


Figure 5.2. T-box transcription factors are necessary for the expansion of antigen-specific NK cells. WT (CD45.1) and Eomes$^{f/f}$ UbCre^{+} (CD45.2, **A**-**B**) or T-bet$^{f/f}$ UbCre^{+} (CD45.2, **C**-**D**) NK cells were co-transferred into *Ly49h*$^{-/-}$ mice and treated with tamoxifen or oil at day -4 PI. Mice were infected with MCMV at day 0 PI. (**A** and **C**) Percentages of Ly49H^{+} NK cells are shown. (**B** and **D**) The relative populations are shown for each time point. (**E**) Assessment of phenotypic markers at day 7 PI for the spleen and liver are shown. (**F**) MFI of KLRG1 staining it day 7 PI in the spleen is shown. (G) Ly49H^{+} NK cells from WT and T-bet$^{f/f}$ UbCre^{+} mice were labeled with CTV, transferred into a *Ly49h*$^{-/-}$ host, and treated with tamoxifen or oil at day -4 PI. Mice were infected with MCMV at day 0 PI. Bar graph shows percentages of divided and non-divided NK cells at day 4 PI for each group. Data are representative of 2-3 experiments with 4-5 mice per condition. * $p < 0.05$ and ns, not significant, paired Student *t*-test.

severely diminished compared to WT controls (Figure 5.2C-D). Unlike T-bet deletion in mature NK cells during homeostasis or homeostatic proliferation (Figure 5.1), virus-specific NK cells lacking T-bet exhibited an increase in the percentage of $TRAIL^+$ NK cells and a more immature phenotype ($CD27^+$ $CD11b^+$ $KLRG1^{lo}$) at day 7 PI (Figure 5.2E-F). A similar phenotype was observed at day 28 PI (data not shown). When deletion of T-bet was initiated at day 3 PI instead of at day -4, similar results were observed (data not shown). These data uncover a novel and stage-specific role of T-bet on NK cells where T-bet promotes TRAIL expression in antigen-inexperienced NK cells but acts to repress TRAIL expression in antigen-experienced NK cells.

To determine if deletion of T-bet in NK cells resulted in decreased expansion due to a difference in proliferation, we adoptively transferred equal numbers of cell trace violet (CTV)-labeled splenic NK cells from WT and $Tbx21^{f/f}$ x $Ub^{Cre\text{-}ERT2}$ mice into $Ly49h^{-/-}$ hosts that were immediately treated with tamoxifen (or oil as a control). Host mice were then infected with MCMV four days post-transfer (PT). Both WT and T-bet-deficient NK cells proliferated after infection, with the WT NK cells undergoing more divisions (Figure 5.2G). Furthermore, when compared to their WT counterparts, a greater percentage of T-bet-deleted NK cells had not divided in response to MCMV infection, suggesting that T-bet expression in mature NK cells is necessary for the execution of a robust proliferation program. Altogether, these data highlight a non-redundant role for T-box transcription factors during NK cell priming for the robust proliferation and proper maturation of virus-specific NK cells.

IL-12 and STAT4 signals upregulate T-bet in NK cells during MCMV infection

To gain insight into when T-box transcription factors may be exerting influences on the proliferation of virus-specific NK cells, the amount of T-bet and Eomes in Ly49H$^+$ NK cells were measured at different time points during MCMV infection. We found that T-bet expression is upregulated at day 2 PI and remains high at day 7 and 42 PI, whereas Eomes expression is largely unchanged immediately following MCMV infection (day 2 PI) and is downregulated in effector and memory NK cells at day 7 and 42 PI, respectively (Figure 5.3A). The rapid modulation of T-bet levels immediately following MCMV infection suggests a role for pro-inflammatory cytokine signals in its upregulation. Because IL-12 has been described to upregulate T-bet expression in CD8$^+$ T cells (23, 118), we evaluated the expression of T-bet in NK cells from WT:*Il12rb2*$^{-/-}$ bone marrow chimeras infected with MCMV. In contrast to WT NK cells, we found that T-bet levels were not greatly upregulated at day 2 and 7 PI in *Il12rb2*$^{-/-}$ NK cells (Figure 5.3B). Eomes expression in *Il12rb2*$^{-/-}$ NK cells did not differ from WT NK cells at various time points during MCMV infection (data not shown).

Given that IL-12 induces T-bet, we predicted that the signal transducer and activator of transcription 4 (STAT4), which acts downstream of the IL-12 receptor, may directly regulate T-bet levels in activated NK cells. Analysis of the *Tbx21* promoter revealed several putative STAT4 binding sites (Figure 5.3C), one of which (site 2) is embedded within a conserved noncoding site (CNS). We performed STAT4 chromatin immunoprecipitation (ChIP) on resting and IL-12/IL-18-activated

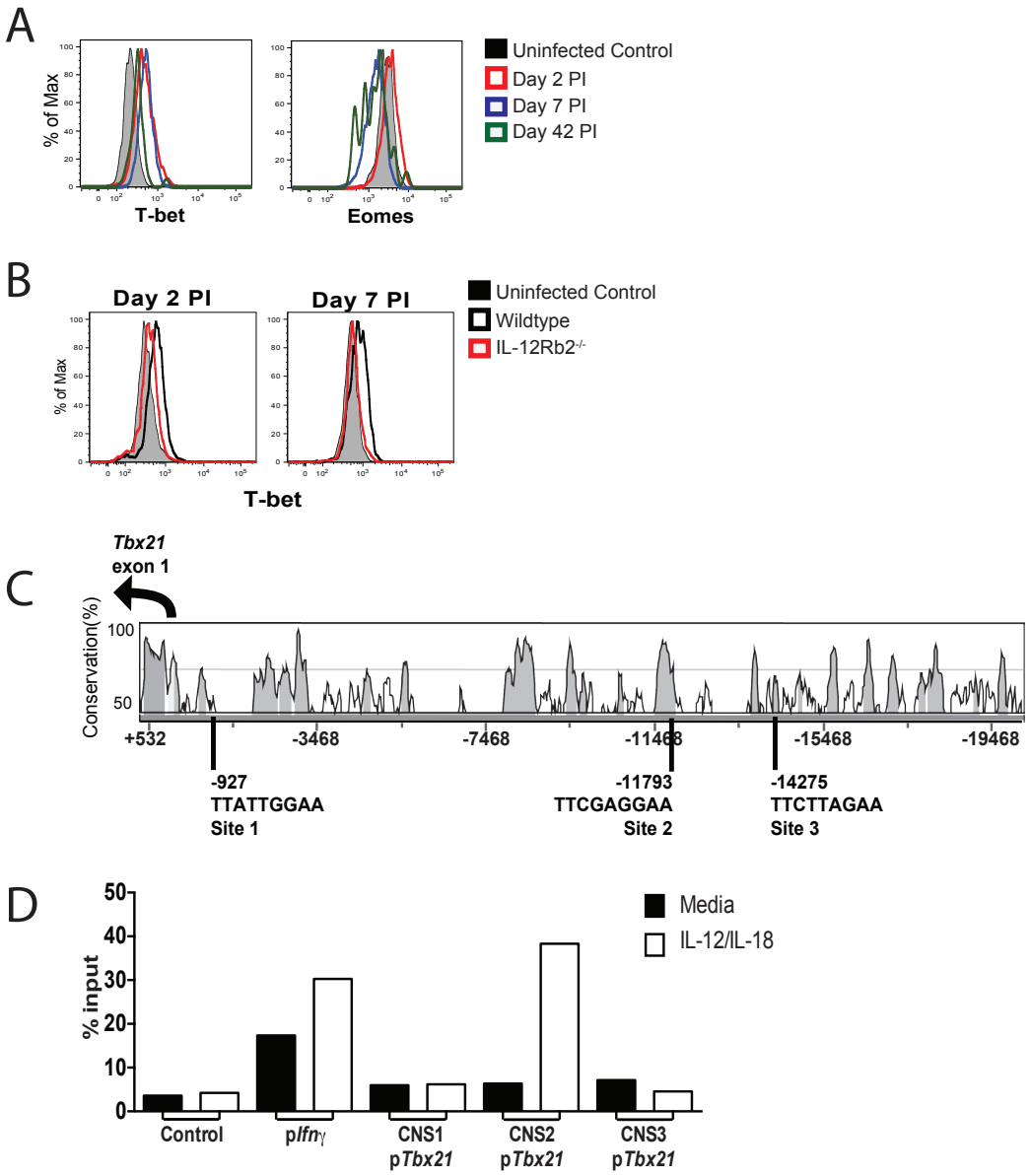


Figure 5.3. IL-12 and STAT4 signals upregulate T-bet expression in NK cells during MCMV infection. (**A**) WT NK cells were transferred into *Ly49h*$^{-/-}$ mice and infected with MCMV. Expression of T-bet and Eomes at the indicated time points is shown. (**B**) Expression of T-bet in WT:*Il12rb2*$^{-/-}$ bone marrow chimeras is shown for the indicated time points. (**C**) Vista browser image of mouse *Tbx21* promoter showing CNS regions (gray shading) and three predicted STAT4 binding sites. (**D**) STAT4 binding at *Tbx21*, *Ifng*, and control promoters as assessed through ChIP followed by qPCR in purified WT NK cells stimulated for 18h with IL-12 and IL-18. STAT4 occupancy as percent of input is shown for target (*Tbx21*) and control DNA (negative control: average of gene desert 50 kb upstream of *Foxp3*, *Utf1*, and *Zfp42* promoters; positive control: *Ifng* promoter). Data is representative of at least three experiments with at least 3 mice per condition.

NK cells, and found substantial enrichment of STAT4 binding at site 2 (Figure 5.3D), ~11 Kb upstream of the *Tbx21* transcriptional start site. Thus, these data support the direct regulation of T-bet expression in NK cells by IL-12 signaling and STAT4 activity during MCMV infection.

T-bet, but not Eomes, is required for the maintenance of virus-specific memory NK cells

We next sought to evaluate the role of Eomes and T-bet on the maintenance of memory NK cells following MCMV infection. We isolated NK cells from the spleen of $Eomes^{f/f}$ x $Ub^{Cre\text{-}ERT2}$ or $Tbx21^{f/f}$ x $Ub^{Cre\text{-}ERT2}$ mice (CD45.2) and co-transferred them with equal numbers of WT NK cells (CD45.1) into $Ly49h^{-/-}$ hosts that were immediately infected with MCMV. At 3 weeks PI, we administered tamoxifen to induce gene deletion. Control mice received oil alone. Given the downregulation of Eomes expression in virus-specific memory NK cells (Figure 5.3A), we were not surprised to find that the ratio of Eomes-deleted to WT NK cells did not change for several weeks after tamoxifen administration (Figure 5.4A).

In contrast to Eomes ablation, induced deletion of T-bet in memory NK cells led to a significant 3 to 4-fold decrease in the ratio of T-bet-deleted to WT cells in mice receiving tamoxifen compared with those receiving oil alone as a control (Figure 5.4B), suggesting a role for T-bet in the persistence of virus-specific memory NK cells. Although no changes were observed in the frequency of TRAIL-expressing or $CD27^+$ $CD11b^+$ NK cells (Figure 5.4C), a significant decrease in the expression of KLRG1 was observed (Figure 5.4D). Altogether, these data uncover a

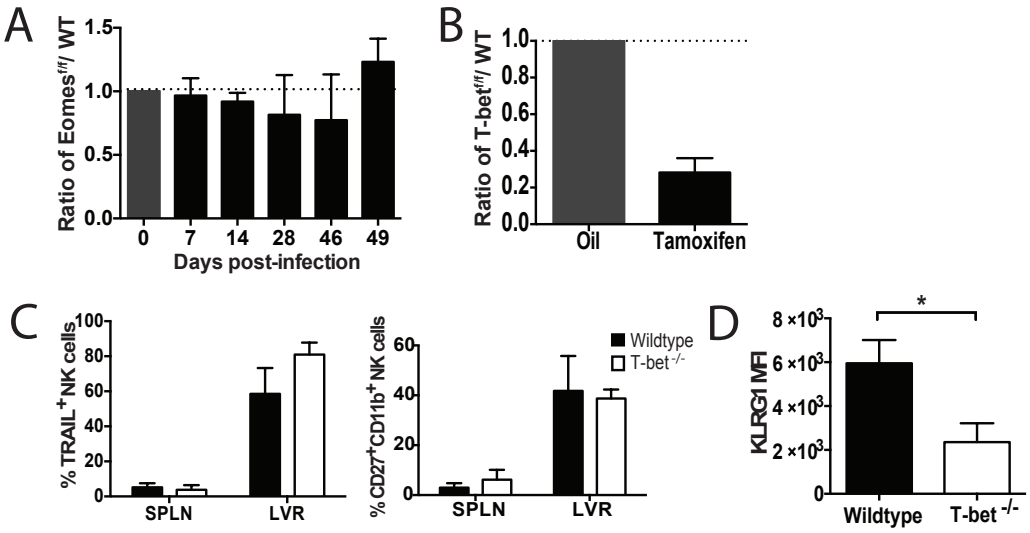


Figure 5.4. T-bet, but not Eomes, is required for the maintenance of memory NK cells. WT (CD45.1) and Eomes$^{f/f}$ UbCre^{+} (CD45.2, **A**) or T-bet$^{f/f}$ UbCre^{+} (CD45.2, **B**-**D**) NK cells were co-transferred into *Ly49h*$^{-/-}$ mice. Mice were infected with MCMV and treated with tamoxifen or oil at day 28s (**A**) or 19 (**B**-**D**) PI. (**A**) The relative fold change of floxed and WT populations relative to their starting ratio is shown. (**B**) The ratio of floxed to WT populations in mice receiving oil versus tamoxifen at day 32 PI is shown. Assessment of phenotypic markers for the spleen and liver (**C** and **D**) are shown. Data is representative of at least three experiments with 4-5 mice per condition. * $p < 0.05$ and ns, not significant, paired Student *t*-test.

unique role for T-bet in the maintenance of number and phenotype of virus-specific memory NK cells, further highlighting the stage-specific function of these T-box family transcription factors.

Discussion

Our understanding of the transcriptional regulation of NK cell development and function has increased during the past decade (119, 120). However, the exact role many of these transcription factors play during the antiviral response of NK cells is largely unknown. T-box family transcription factors Eomes and T-bet have overlapping and distinct effects on NK cell maturity and function. Eomes and T-bet were previously shown to play a redundant role in the induction of CD122, the receptor that mediates IL-15 signaling and promotes the survival and homeostasis of NK cells and memory T cells (58). During NK cell development, Eomes has been described to support NK cell maturation past the $DX5^+$ stage, a step that is characterized by acquisition of a full Ly49 repertoire (61). T-bet appears to direct the development of $Eomes^-$ NK cells in the liver (62), and $TRAIL^+$ NK cells (61, 121). Although the importance of T-bet and Eomes in NK cell development has been investigated, their influence on the antigen-specific NK cell response has not been characterized. Using an inducible deletion system, we have evidence to support a stage-specific and non-redundant role for T-box transcription factors during NK cell homeostasis, homeostatic proliferation, antiviral response, and generation of long-lived memory.

Deletion of Eomes or T-bet before or early during infection led to a significant decrease in the expansion of *Eomes*$^{f/f}$ *x Ub*$^{Cre\text{-}ERT2}$ or *Tbx21*$^{f/f}$ *x Ub*$^{Cre\text{-}ERT2}$ NK cells relative to WT. This could be due to possible complementary actions of Eomes and T-bet on the promotion of a proliferative program following infection. T-bet-deficient NK cells exhibited a defect in proliferation following MCMV infection, thus highlighting the importance of this transcription factor on the execution of a robust proliferative program. Studies exploring the effect Eomes expression has on NK cell proliferation are currently underway.

We found the expression of TRAIL to be more fluid than previously thought. Despite TRAIL$^+$ DX5$^-$ NK cells being described to originate in the fetal liver (122), we found deletion of T-bet early during MCMV infection resulted in an increased frequency of TRAIL$^+$ NK cells. This increase in the TRAIL$^+$ population was accompanied by a decrease in maturity (higher percentages of CD27$^+$ x CD11b$^+$ cells and less KLRG1 expression). This reliance on T-bet for the maintenance of a mature phenotype was also observed following the deletion of T-bet in memory NK cells. Previous reports have described T-bet as an important factor in the terminal maturation of NK cells (60). Our data suggest that T-bet is needed continuously for the maintenance of a mature phenotype.

Although Eomes and T-bet may have complementary functions in the induction of a proliferative program in response to infection, T-bet acts to maintain antigen-experienced NK cells in a mature state. Current studies exploring the effect of Eomes expression on the phenotype of activated NK cells are underway. We propose that in addition to TRAIL marking the distinction of immature NK cells

(61, 122) and a possibly distinct NK cell lineage in the liver (62), it may also mark a more immature population, as loss of TRAIL^{+} was accompanied with increased KLRG1 expression. Interestingly, deletion of T-bet at a memory time point skewed a previously equal WT: *Tbx21*$^{f/f}$ *x Ub*$^{Cre\text{-}ERT2}$ population in favor of WT, suggesting that T-bet may be necessary in the maintenance of memory NK cells. This finding could signify a stage-specific effect for T-bet where it functions to maintain TRAIL expression and a mature phenotype in naïve NK cells but promotes persistence and maturity, repressing TRAIL expression, in virus activated antigen experienced NK cells. Future studies will address the effect loss of T-bet and Eomes may have on recall responses.

The impact of Eomes and T-bet in antigen-experienced NK cells at various stages during viral infection highlights the continuous importance of these transcription factors throughout the lifespan of NK cells. Future studies will investigate the specific gene targets of Eomes and T-bet in resting, activated, and memory NK cells, in order to further understand their distinct or complementary activity during the antiviral NK cell response. Our current findings reveal distinct molecular events that control virus-specific NK cell responses, and may inform clinical approaches that couple NK cell-mediated immunity to the treatment of human diseases.

Materials and Methods

Mice, tamoxifen treatment, and infections

All of the mice used in this study were bred and maintained at MSKCC in accordance with IACUC guidelines. Mixed bone marrow chimeric mice were generated and adoptive transfer studies were performed as previously described (13). Mice were infected by intraperitoneal (IP) injections of MCMV (Smith strain) with 7.5×10^3 plaque-forming units. Mice were administered 8 mg tamoxifen dissolved in 200 μL olive oil by oral gavage following a day 0, 1, 3 regimen. Control mice received 200 μL olive oil.

Flow cytometry and cell sorting

Fc receptors were blocked with 2.4G2 mAb before staining with the indicated surface or intracellular antibodies (BD, BioLegend, or eBioscience). Flow cytometry was performed on an LSR II (BD). Cell sorting was performed on an Aria II cytometer (BD). All data were analyzed with FlowJo software (TreeStar). NK cell enrichment and adoptive transfers were performed as previously described (91).

qRT-PCR and ChIP

*Quantitative reverse-transcription PCR and chromatin immunoprecipitation (ChIP) were performed as described previously (92). The following qPCR primers were used for ChIP studies: CNS1 p*Tbx21, *For: 5'- CTAAGCAGGCACTCCATCAGTTG*

*-3', Rev: 5'- GTCCTTCCTCCGCTGTTCTATTC -3'; CNS2 p*Tbx21, *For: 5'-TAGCGGAAAGCGAGATGGTG -3', Rev: 5'-AGTGAAGGAGTTCTGTGGTTCTGG -3'; CNS3 p*Tbx21, *For: 5'-GAGCCGACATACTGACATTCTGC -3', Rev: 5'-CATTCTCCTCTCCCACCATCTTG -3';* Ifng promoter, *For: 5'-GCTCTGTGGATGAGAAAT-3', Rev: 5'-GCTCTGTGGATGAGAAAT-3'; Gene desert 50 kB upstream of* Foxp3*, For: 5'-TAGCCAGAAGCTGGAAAGAAGCCA-3', Rev: 5'-TGATACCCTCCAGGTCCAACCATT-3';* Utf1, *For: 5'-AGTCGTTGAATACCGCGTTGCTG-3', Rev: 5'-CTGTTGAGATGTCGCCCAAGTGC-3';* Zpf42*, For: 5'-AGAGGGCGGTGTGTACTGTGGTG-3', Rev: 5'-CTTCTTCTTGCACCCGGCTTGAG-3'.*

Statistical methods

All plots depict mean ± s.e.m. Two-tailed unpaired Student's *t*-test was used to derive statistical differences. A p value > 0.05 was considered significant. Plots and statistical analyses were produced in GraphPad Prism.

CHAPTER 6

CONCLUSION

Traditionally classified as a member of the innate immune response, NK cells continue to challenge the conceptual boundaries delineating the innate and adaptive immune compartments. Although immunological memory has been a defining pillar for cells of the adaptive immune response, recent work has uncovered this characteristic in NK cells. NK cells are able to respond to certain small molecules and viruses in an antigen-specific manner, and mount an enhanced response upon antigen reencounter. Because the precise signals governing NK cell "memory" were largely undefined, this thesis work explored the role of pro-inflammatory cytokines IL-12, IL-18, and type I IFN, and T-box transcription factors, T-bet and Eomes, on the generation of a long-lived anti-viral NK cell response.

Similar to the need for "Signal 3" by $CD8^+$ T cells, NK cells can now be appreciated to rely on pro-inflammatory cytokine signals for a productive antiviral response. The intricate details of "Signal 3" for NK cells are summarized in Figure 6.1. In conjunction with Ly49H engagement and costimulation through CD28 triggering, IL-12 signaling leads to the activation and dimerization of STAT4, which translocates into the nucleus to promote IFN-γ production and the proliferation of Ly49H-bearing NK cells. The nuclear translocation of activated STAT4 dimers also leads to the increase in expression of MyD88 and T-bet. Increased levels of Myd88 potentiate IL-18 signaling, and result in increased IFN-γ

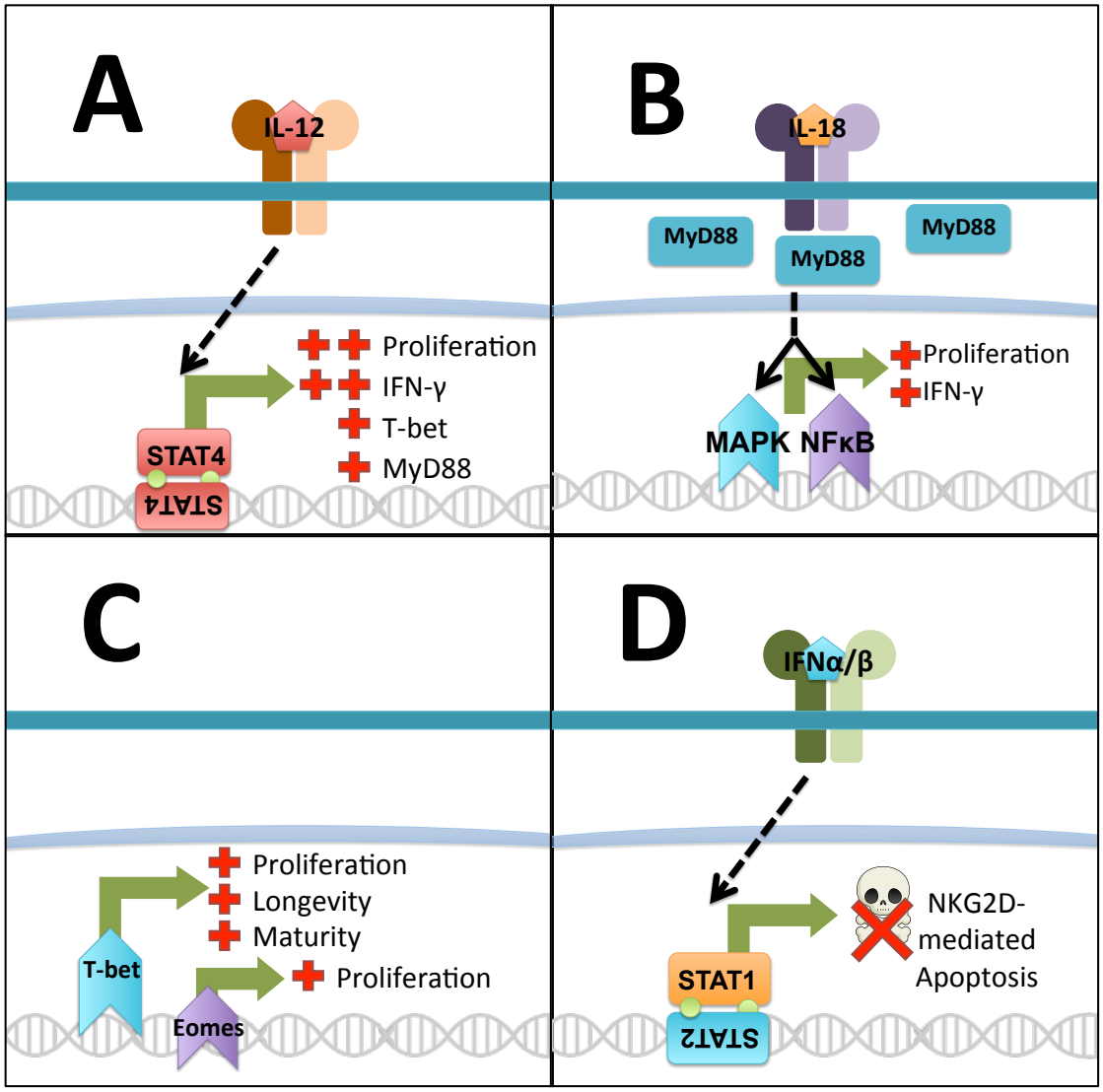


Figure 6.1. Pro-inflammatory cytokine and transcription factor control of the anti-viral NK cell response. In conjunction with Ly49H engagement and costimulation through CD28 triggering, **(A)** IL-12 signaling leads to the activation and dimerization of STAT4, which translocate into the nucleus to increase proliferation, IFN-γ production, and the expression Myd88 and T-bet. **(B)** Increase of Myd88 potentiates IL-18 signaling, and results in increased IFN-γ production and proliferation. **(C)** T-bet and its close family member, Eomes, act in a non-redundant fashion to promote the anti-viral proliferative program. Constant T-bet expression is necessary at all stages of the activated NK cell's lifecycle to maintain a mature phenotypic program, as well as in memory NK cells to promote cellular persistence. **(D)** Lastly, type I IFN signaling acts to protect the antiviral NK cell response from NKG2D-mediated apoptosis by inhibiting the upregulation of NKG2D-ligand expression.

production. T-bet and its close family member, Eomes, act in a non-redundant fashion to promote the anti-viral proliferative program. Furthermore, constant T-bet expression is necessary at all stages of the NK cell response to maintain a mature phenotypic program, as well as in memory NK cells to promote cellular survival. Lastly, type I IFN signaling acts to protect the antiviral NK cell response from NKG2D-mediated apoptosis. All of these events act in concert to promote the long-lived antiviral NK cell response. These findings further our understanding of the complex network of pro-inflammatory cytokine signaling on the different stages of the NK cell response. The capability of an NK cell to kill a susceptible target without prior sensitization, in addition to their role in the control of viral infections, has made this cell an attractive target in vaccine strategies and anti-tumor immunotherapy. Incorporation of pro-inflammatory cytokine treatment, or targeting of transcription factors in NK cells, can be beneficial in harnessing NK cell function for therapeutic purposes.

Pro-inflammatory cytokines act on NK cells to mediate an effect on the shaping of the immune response, creating a bridge that functionally connects innate immunity and adaptive immunity. NK cells and dendritic cells (DC) are known to extensively cross-talk (123, 124), with pro-inflammatory cytokines mediating a significant role in this NK-DC communication. This thesis work elaborates on the DC-derived pro-inflammatory cytokines that shape the NK response. Reciprocally, NK cells promote DC maturation and participate in the maturation of DCs (124). It has been shown that NK cell-mediated DC maturation is dependent on pro-inflammatory cytokines IL-12 (125), IL-18 (126), and type I IFN (127). NK cells

"edit" mature DC populations through direct killing of immature DCs (127-129). This form of DC "editing" by NK cells can be seen as a method of "quality control", where only properly activated DC are allowed to migrate to the lymph nodes and subsequently activate a productive T cell response. Furthermore, NK cell-mediated killing of infected cells provides antigen-presenting cells, like the DC, with the apoptotic bodies necessary to prime a CTL response (127, 129-131).

The reciprocity of NK-DC interactions has led to the direct targeting of NK cells in DC-based vaccine strategies (18). Vaccines where DCs are modified to express cytokines such as IL-12 with and without IL-15 resulted in the eradication of mouse tumors in an NK cell- and T cell-dependent fashion (132-135). Furthermore, in a mouse model of neuroblastoma, a fusion vaccine consisting of DC and neuroblastoma tumor cells showed a complete protective effect against liver metastases in mice that received fused cells transduced to express both IL-12 and IL-18 (136). The protective effect of this fusion vaccine was shown to correlate with NK cell and T cell activation. Therefore, pro-inflammatory cytokines can be used to target the NK cell compartment in the context vaccination. Furthermore, given the role of T-bet expression in the maintenance of memory NK cells, targeting transcription factors in vaccination strategies may prove fruitful in boosting activated NK cell numbers. A combinatorial vaccination approach that incorporates the effects of multi-cytokine treatment and transcription factor expression will prove paramount to vaccine development.

Cancer immunotherapy has seen some success with the use of systemic and direct-immune cell pro-inflammatory cytokine treatment. However, in the case of

systemic cytokine administration, toxicities from the high doses necessary for therapeutic effects remain a limiting factor (137). For instance, high dose type I IFN administration has been used in the treatment of metastatic malignant melanoma with low response rates and high toxicities (138-140). Furthermore, systemic IL-12 administration can lead to serious hematologic and hepatic toxicities (141). Despite these side effects, certain studies show multi-cytokine therapy has the potential to increase the positive benefits of NK cell anti-tumor function, while simultaneously decreasing toxicity of treatment. Similar to MCMV, where IL-12 potentiates IL-18 signaling through the increase of MyD88 (Chapter 3), *in vivo* anti-tumor cytokine treatments have suggested inter-cytokine pathway modulation. In a mouse model of malignant melanoma, pretreatment with IL-12, followed by low dose type I IFN, significantly increased survival of tumor-bearing mice (142). IL-12 pre-treatment sensitized tumors and host immune effector cells to low dose type I IFN administration through the increase of STAT1, STAT2, and IFN regulatory factor 9 (IRF9). This effect was mediated by IL-12-elicited IFN-γ production. This study identifies an indirect mechanism through which IL-12 modulates another cytokine signaling pathway at the level of signal transduction and gene regulation. Multi-cytokine therapy can also exhibit synergy in promoting anti-tumor effects and NK cell activation. In a mouse head and neck cancer model, cytokine gene therapy using type I IFN in combination with IL-12 or IL-2 resulted in augmented tumor inhibition, and activation of NK cells and $CD8^+$ T cells (143). A greater appreciation of cytokine pathways and their potential interactions, direct and indirect, is warranted.

Direct pro-inflammatory cytokine treatment of NK cells can also harness NK cell effector functions towards cancer immunotherapy. Cytokine treatment of mice and human naïve NK cells results in a prolonged lifespan and enhanced effector functions upon restimulation, features reminiscent of memory (25-27). Consequently, cytokine treated NK cells were appropriately termed cytokine-induced memory-like (CIML) NK cells. Studies in mice have shown CIML NK cells are more protective than untreated NK cells against established tumors (26). However, the mechanisms through which pro-inflammatory cytokine treatment leads to CIML NK cell formation are unknown. Our current work may provide a better understanding of the mechanisms through which pro-inflammatory cytokines exert their effects on NK cell function. For instance, Chapter 5 describes the ability of IL-12 to increase T-bet expression during MCMV infection. Both T-bet and IL-12 signaling in NK cells leads to increased proliferation and longevity, and IL-12 is a commonly used cytokine in the generation of CIML NK cells. Could IL-12-dependent proliferation and longevity function through a modification of the T-bet gene locus that keeps it more accessible to transcription in memory NK cells? Furthermore, Chapter 3 describes the stage-specific role of IL-18 on NK cell proliferation, where IL-18 is needed for the naïve but not the memory NK cell proliferative response. How does IL-18 signaling modify the proliferative pathways of an activated NK cell? Can the epigenetic landscape of naïve and memory/CIML NK cells explain differences in phenotype and function? Further investigation of these cytokine and transcription factor effects on CIML NK cells may prove key in enhancing NK cell functions and longevity. Moreover, the memory-like qualities of

NK cells, such as enhanced effector functions and longevity, are retained by daughter cells, suggesting cytokine-induced changes are hereditary.

Recent studies in humans have described an epigenetic repatterning in adaptive or "memory" NK cell population associated with HCMV seropositivity and latent HCMV reactivation (144-146). Epigenetic diversification of adaptive NK cells correlated with genome-wide DNA methylation repatterning, which shared ample similarity with cytotoxic T cells but differed from canonical NK cells (145). These epigenetically unique adaptive NK cells also possessed distinct functionalities when compared to canonical NK cells, thus suggesting epigenetic diversification as a mechanism in the generation of a memory-like NK cell pool. Although these studies failed to investigate the precise signals that led to epigenetic repatterning, knowing the heritable and likely epigenetic changes induced by cytokine treatment will be of importance to the advancement of NK cell immunotherapy. Enhanced molecular approaches will be necessary to decipher the changes endured at the gene expression level that lead to a memory NK cell.

NK cells also mediate antibody-dependent cellular cytotoxicity (ADCC) through the recognition of antibody-coated targets by the activating receptor, CD16 (Fcγ RIIIa) (147, 148). ADCC accounts for an important part of the efficacy shown by Rituximab (anti-CD20, non-Hodgkin's lymphoma) and Herceptin (anti-HER2, metastatic breast cancer and gastric carcinoma) treatment of patients (149, 150). Cytokine treatment can increase ADCC. IL-12, IL-18, IL-2 and IL-21 are documented to increase ADCC in combination with specific antibodies (151, 152). Furthermore, type I IFN is implicated in promoting Rituximab-mediated ADCC in a

model using IL-15 and CpG ODN A stimulation of PBMC (153). CpG ODN A is a potent inducer of type I IFN (154); therefore, the direct function of type I IFN on ADCC warrants investigation. In addition to cytokine treatment, co-stimulatory receptor triggering has been shown to increase NK cell-mediated ADCC. NK cells stimulated by anti-4-1BB antibodies regressed subcutaneous lymphoma tumors in mice receiving treatment with Rituxumab (155). Eomes has been shown to be required for 4-1BB-mediated antitumor effects (156), which suggests that co-stimulation pathways in NK cells may utilize members of the T-box transcription factor family to mediate their function. The effects of co-stimulation in NK cells are not well known, however, the role of T-box transcription factors in mediating costimulatory molecule-induced or ADCC-mediated effector functions remains of interest.

Antibody-cytokine fusion proteins, referred to as immunocytokines (IC), represent a promising way to reap the benefits of cytokine and antibody treatment against certain tumors while minimizing cytokine-mediated toxicity. ICs enhance cytokine and Fc binding, resulting in greater conjugate formation between the NK cell and the antibody-coated tumor cell (157). Improvement of synapse formation results in greater NK cell effector functions and anti-tumor effect, when compared to the separate administration of antibody with equivalents amount of cytokines (150, 157). ICs have the potential to improve the therapeutic index of cytokines by concentrating their function at the site of localized or disseminated disease, thus reducing unwanted side effects often associated with systemic cytokine administration.

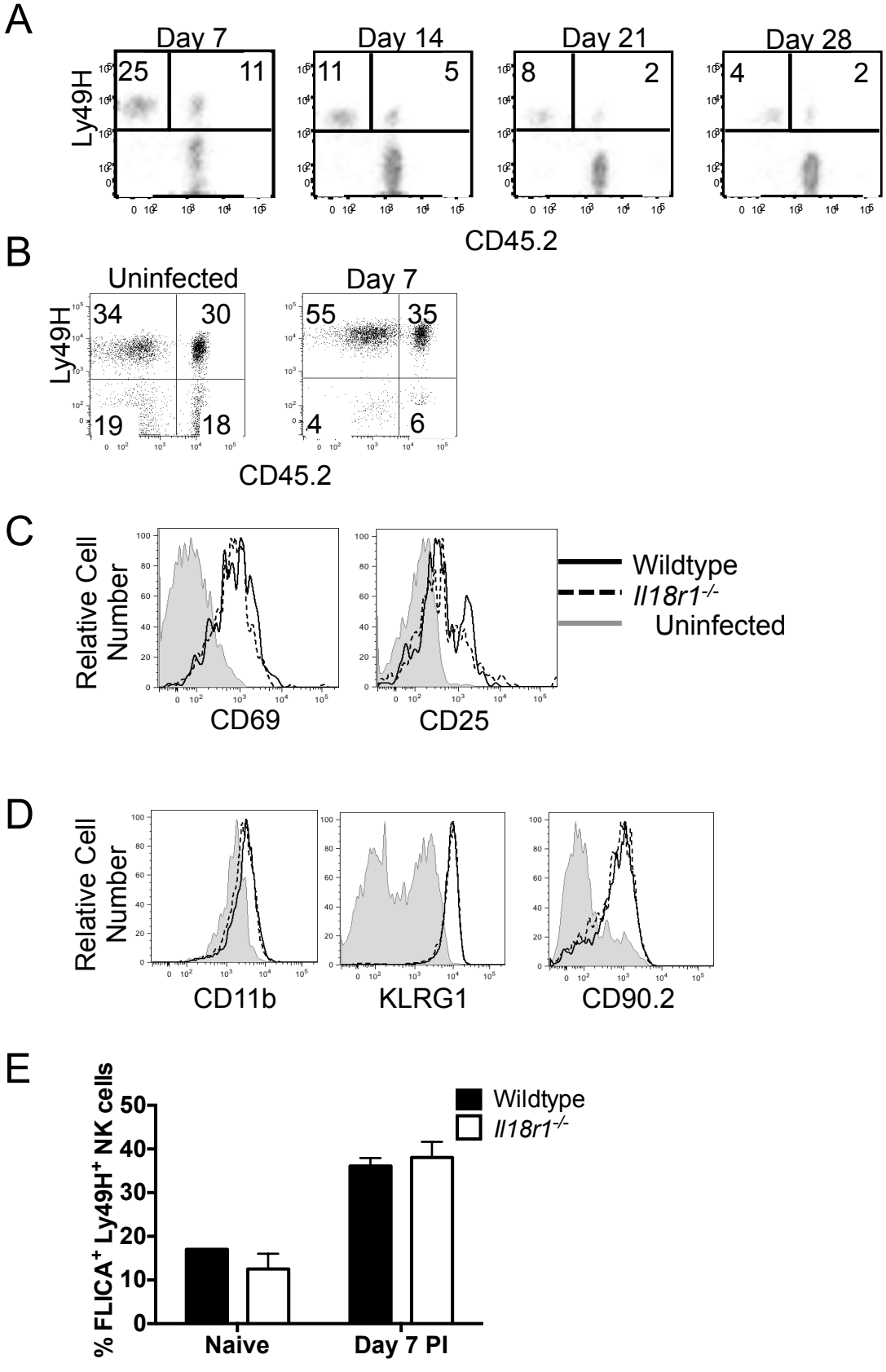
Although pro-inflammatory cytokine treatment of NK cells results in the activation, proliferation, and attainment of effector function, the trafficking of NK cells to a desired location remains an issue. Localized cytokine function, such as with the use IC treatments, can circumvent difficulties in targeting activated NK cells to the area of need (tumor or infection). Exploring the effect of cytokines or transcription factors on the expression of NK cell trafficking receptors, like that of IL-18 on CCR7 expression in human NK cells (49) or T-bet on S1P5 expression in mouse NK cells (158), may help improve the efficacy of NK cell use in therapeutic strategies.

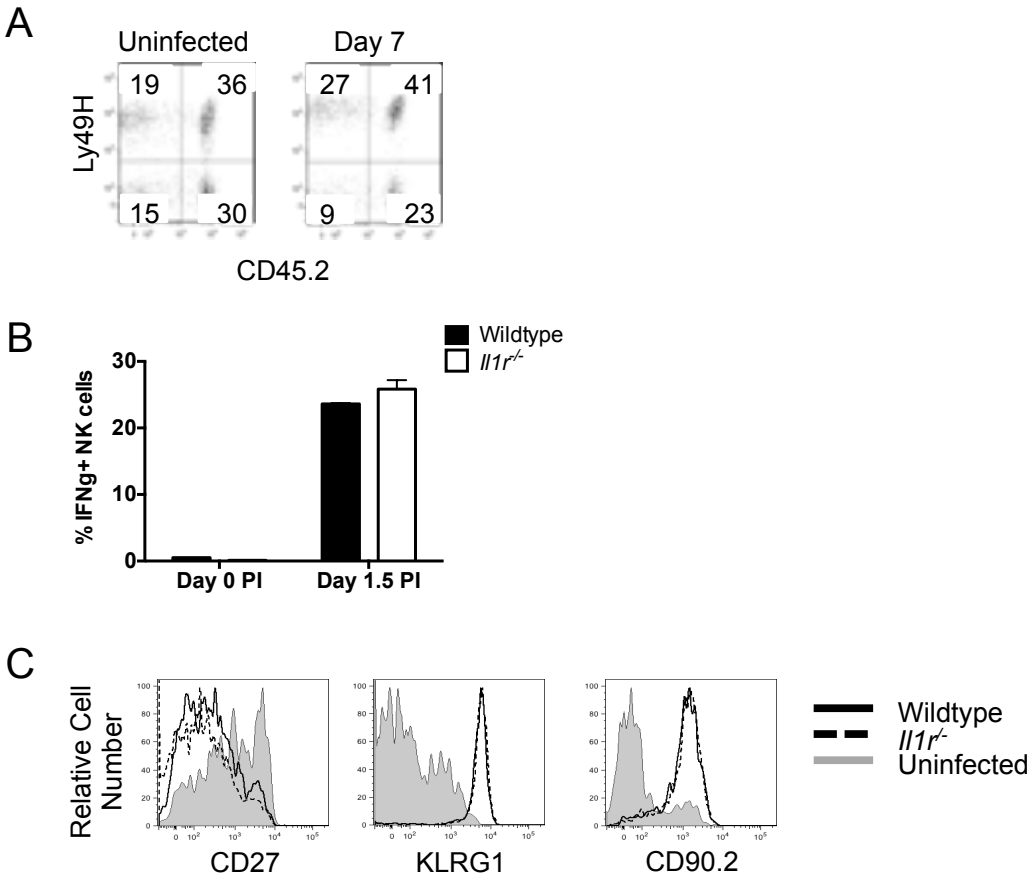
Attaining a better understanding of what molecular signals execute specific NK cell functions is paramount to our ability to harness their actions for therapeutic purposes. Pro-inflammatory cytokines IL-12, IL-18, and type I IFN, and transcription factors, T-bet and Eomes, affect multiple aspects of the immune response. This thesis work expands our understanding of the complex network of pro-inflammatory cytokine signaling on the different stages of the NK cell response.

APPENDIX 1

SUPPLEMENTARY FIGURES FOR CHAPTER 3

Supplementary Figure 3.1. IL-18R-deficient NK cells exhibit expansion defect following MCMV infection. (**A**) 10^6 WT (CD45.1) and *Il18r1*$^{-/-}$ (CD45.2) NK cells were co-transferred into Ly49H-deficient mice (CD45.2) and infected with MCMV. Plots are gated on total NK cells and the percentages of Ly49H$^+$ NK cells are shown for each time point PI. (**B**) WT (CD45.1) and *Il18r1*$^{-/-}$ (CD45.2) mixed bone marrow chimeric mice were infected with MCMV and percentages of splenic NK cells are shown (gated on TCR-β^- NK1.1$^+$) for uninfected and day 7 PI. Expression of (**C**) CD69, CD25 at day 1.5 PI and (**D**) CD11b, KLRG1, and CD90.2 at day 7 PI are shown for wildtype and *Il18r1*$^{-/-}$ Ly49H$^+$ NK cells (compared to uninfected mice) in mixed bone marrow chimeric mice. All data are representative of three experiments with 2-3 mice per time point. (**E**) Day 7 splenocytes were incubated with FLICA® FAM-VAD-FMK and percentages of Ly49H$^+$ FLICA$^+$ NK cells are shown.


A
Day 7
Day 14
Day 21
Day 28
Ly49H
25
11
11
5
8
2
4
2
CD45.2
B
Uninfected
Day 7
Ly49H
34
30
19
18
55
35
4
6
CD45.2
C
Relative Cell Number
CD69
CD25
Wildtype
Il18r1-/-
Uninfected
D
Relative Cell Number
CD11b
KLRG1
CD90.2
E
% FLICA+ Ly49H+ NK cells
50
40
30
20
10
0
Naive
Day 7 PI
Wildtype
Il18r1-/-



Supplementary Figure 3.2. NK cell expansion and memory formation are independent of IL-1 during MCMV infection. (**A**) Wildtype ($CD45.1^+$) and *Il1r*$^{-/-}$ ($CD45.2^+$) mixed bone marrow chimeric mice were infected with MCMV and percentages of splenic NK cells are shown (gated on TCR-β^- $NK1.1^+$) for uninfected mice and at day 7 post-infection. (**B**) Percentages of IFN-γ^+ wildtype and *Il1r*$^{-/-}$ NK cells are shown at day 0 and 1.5 PI. (**C**) Expression of CD27, KLRG1, and CD90.2 are shown for wildtype and *Il1r*$^{-/-}$ $Ly49H^+$ NK cells (compared to uninfected mice) at day 7 PI. All data are representative of two independent experiments with 3-4 mice per time point.

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